The effects of synuclein null mutations on murine physiology and development

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DECLARATION

I hereby declare that the composition of this thesis and the findings presented in it are entirely my own work with the exception of the assay of dopamine and its metabolites in synuclein null mutant mice which was carried out by Dr John Sharkey and Dr Paul Jones of the Fujisawa Research Institute, Edinburgh. Some of this work has been published in abstract and paper form.

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Publications

Robertson DC, Schmidt O, Ninkina N, Jones PA, Sharkey J, Buchman VL (2004) Developmental loss and resistance to MPTP toxicity of dopaminergic neurones in substantia nigra pars compacta of gamma-synuclein, alpha-synuclein and double alpha/gamma-synuclein null mutant mice. J Neurochem 89(5): 1126-36. **(see Appendix).**

Ninkina N, Papachroni K, Robertson DC, Schmidt O, Delaney L, O'Neill F, Court F, Rosenthal A, Fleetwood-Walker SM, Davies AM, Buchman VL (2003) Neurons expressing the highest levels of gamma-synuclein are unaffected by targeted inactivation of the gene. Mol Cell Biol 23(22): 8233-45**. (see Appendix).**

Robertson DC, Schmidt O, Ninkina N, Davies AM, Fleetwood-Walker SM, Middleton G, Buchman VL (2002) Developmental deficit of dopaminergic neurons in γ-synuclein null mutant mice. Program No. 484.16. *2002 Abstract Viewer/Itinerary Planner.* Washington, DC: Society for Neuroscience, Online.

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Chapter 1

ABSTRACT

The synuclein family came into the limelight with the discovery of two point mutations in the human α-synuclein gene associated with autosomal dominant, familial Parkinson's disease. This triggered numerous studies aimed at understanding the mechanism of α-synuclein involvement in neurodegeneration. Fibrillated α-synuclein has been identified as a major component of Lewy bodies in dopaminergic neurones of the substantia nigra pars compacta (SNpc) in cases of idiopathic Parkinson's disease as well as other diseases in which inclusions feature now known as synucleinopathies. Moreover, it has been demonstrated that α-synuclein can aggregate in vitro into filaments structurally similar to filaments found in pathological inclusions and that mutated forms of human α-synuclein aggregate substantially faster than wild-type protein. These results suggested a causative role of α-synuclein aggregation in the development of synucleinopathies.

We studied the effects of a targeted inactivation of γ-synuclein and later α-synuclein and α/γ-synuclein together on murine physiology and development. These animals are viable and fertile with no gross physiological of morphological abnormalities. A quantitative evaluation of the substantia nigra showed a reduction in the number of dopaminergic neurones in the SNpc region but not in ventral tegmental area (VTA) of adult γ-synuclein null mutant mice. Similar reductions were revealed in α-synuclein and double α/γ-synuclein null mutant animals, this difference appearing after birth and remaining constant throughout life. However, in none of these mutants did this lead to significant changes in levels of striatal dopamine or dopamine metabolite levels or motor function.

In all three studied types of null mutants, dopaminergic neurones of SNpc were resistant to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) toxicity. We propose that both synucleins are important for effective survival of some SNpc neurones during a critical period of development and that, in the absence of these proteins, permanent activation of compensatory mechanisms allow many neurones to survive and become resistant to certain toxic insults.

We have shown that the absence of α-, γ- or α- and γ-synuclein does not precipitate any obvious or serious developmental or functional abnormalities. If the synuclein family are important for the correct development of the SNpc then compensatory mechanisms exist to limit the effect of their absence, not giving rise to any visible deleterious phenotype.

ABBREVIATIONS

Ab	antibody
AD	Alzheimer's disease
ATP	adenosine-tri-phosphate
AX	axonal
BCSG-1	breast specific cancer gene-1
BG	basal gangla
CB	cell bodies
C-terminal	carboxy terminal
CaM kinase II	calcium/calmodulin dependent protein kinase II
CNS	central nervous system
DA	dopamine
DAB	3,3 di-amminobenzidine
DAT	dopamine transporter
DLB	dementia with Lewy bodies
DRG	dorsal root ganglia
E(number)	embryonic day (number)
ER-α	oestrogen receptor-α
ERK	extracellular signal regulated kinase
ES	embryonic stem
GABA	gamma-amino butyric acid
GAPDH	glyceraldehydes-3-phosphate dehydrogenase
GCI	glial cytoplasmic inclusion

GDNF	glial cell-line derived neurotrophic factor
GNI	glial nuclei inclusion
GST	glutathione S-transferase
HPLC	high performance liquid chromatography
HSP	heat shock protein
HRP	horse radish peroxidase
HSS	Hallervorden-Spatz syndrome
IHC	immunohistochemistry
LB	Lewy body
MPTP	1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MPPP	1-Methyl-4-phenyl-4-propionoxypiperidine
MPP^+	1-methyl-4-phenylpyridinium
$MPDP^+$	1-methyl-4-phenylpyridinium
MSA	multiple system atrophy
N-terminal	amino terminal
NACP	non-amyloid component of senile plaques precursor protein
NAI	neuronal axon inclusion
NBF	neutral buffered formalin
NBIA-1	neurodegeneration with brain iron accumulation type-1
NCD	natural cell death
NCI	neuronal cytoplasm inclusion
NGF	nerve growth factor
NNI	neuronal nuclei inclusion
OPAC	sporadic olivopontocerebellar atrophy

P(number)	postnatal day (number)
PANK2	pantothenate kinase gene
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PD	Parkinson's disease
PFA	paraformaldehyde
PINK1	PTEN-induced putative kinase-1
PKA	protein kinase A
PKAN	pantothenate kinase-associated neurodegeneration
PKC	protein kinase C
PLD2	phospholipase D2
PNP-14	phosphoneuroprotein-14
PNS	peripheral nervous system
RBD	REM sleep behaviour disorder
REM	rapid eye movement
RNA	ribonucleic acid
ROS	reactive oxygen species
rpm	revolutions per min
SDS	Shy-Drager Syndrome
SN	*substantia nigra*
SNCA	α-synuclein gene
SNCB	β-synuclein gene
SNCG	γ-synuclein gene
SNpc	substantia nigra *pars compacta*

SNpr	substantia nigra *pars reticulata*
TG	trigeminal ganglia
TH	tyrosine hydroxylase
TM	transmembrane
UCHL-1	ubiquitin C-terminal hydrolase L1
VTA	ventral tegmental area
WT	wild-type

Chapter 1: INTRODUCTION

This thesis chronicles the work undertaken over the course of a postgraduate research project funded by the Medical Research Council. The focus is a family of small proteins, the synucleins, implicated in several neurodegenerative pathologies now classified together as the synucleinopathies. This is an attempt to elucidate potentially valuable information relating to their physiological function.

1.1 History of the synuclein family

1.1.1 Synucleins

Synucleins are small proteins (123-143 amino acids) characterised by imperfect (KTKEGV) repeats distributed throughout the highly conserved amino-terminal half of the polypeptide. Synucleins have a more diverse acidic carboxyl-terminal (C-terminal) region possessing very little homology and are expressed exclusively in vertebrates (Fig. 1.1).

The first synuclein was cloned from the electric ray *Torpedo Californica* by screening an expression library with an antiserum raised against cholinergic vesicles (Maroteaux, Campanelli and Scheller, 1988). This protein was named synuclein due to its initial localisation within the presynaptic nerve terminal and neuronal nuclei. There are three identified synuclein proteins now known as α-, β- and γ-synuclein. These are a family of natively unfolded proteins, which have little or no ordered tertiary structure under physiological conditions, characterised by a unique combination of low overall hydrophobicity and large net charge (Weinreb, Zhen, Poon, Conway, Lansbury, 1996; Uversky, Li and Fink, 2001).

α-synuclein first began to attract attention in 1993 with the discovery that a 35 amino acid fragment of the so-called non-Aβ component precursor protein (NACP) was present in the senile plaques found in Alzheimer's disease patients (Table 1.1) (Ueda, Fukushima, Masliah, Xia, Iwai, Yoshimoto, Otero, Kondo, Ihara and Saitoh, 1993). Subsequently, the avian ortholog of α-synuclein, synelfin, was described as being up-regulated during the critical phase of song learning in the zebra finch, leading to the hypothesis that this synuclein was involved in neuronal plasticity (George, Jin, Woods and Clayton, 1995). Interest intensified with the discovery that a mutation at the α-synuclein locus was present in families displaying inherited Parkinson's disease. This mutation produces an alanine-53-threonine (A53T) amino acid substitution in the encoded protein. This was found to be present in Italian kindred and three unrelated Greek families suffering early onset familial Parkinson's disease (PD) (Polymeropoulos, Lavedan, Leroy, Ide, Dehejia, Dutra, Pike, Root, Rubenstein, Boyer, Stenroos, Chandrasekharappa, Athanassiadou, Papapetropoulos, Johnson, Lazzarini, Duvoisin, Di Iorio, Golbe and Nussbaum, 1997). A second mutation, alanine-30-proline (A30P), was also reported to be associated with families displaying this rare inherited form of the disease (Kruger, Kuhn, Muller, Woitalla, Graeber, Kosel, Przuntek, Epplen, Schols and Riess, 1998). The pathological significance of these mutations will be discussed in greater detail below, suffice to say that synucleins have become of great interest due to their involvement in a wide range of neurodegenerative disorders.

1.1.2 α-synuclein

The α-synuclein gene (SNCA) has been localised to chromosome 4q12.3-q22 and contains seven exons, five of which are highly conserved coding exons (Campion,

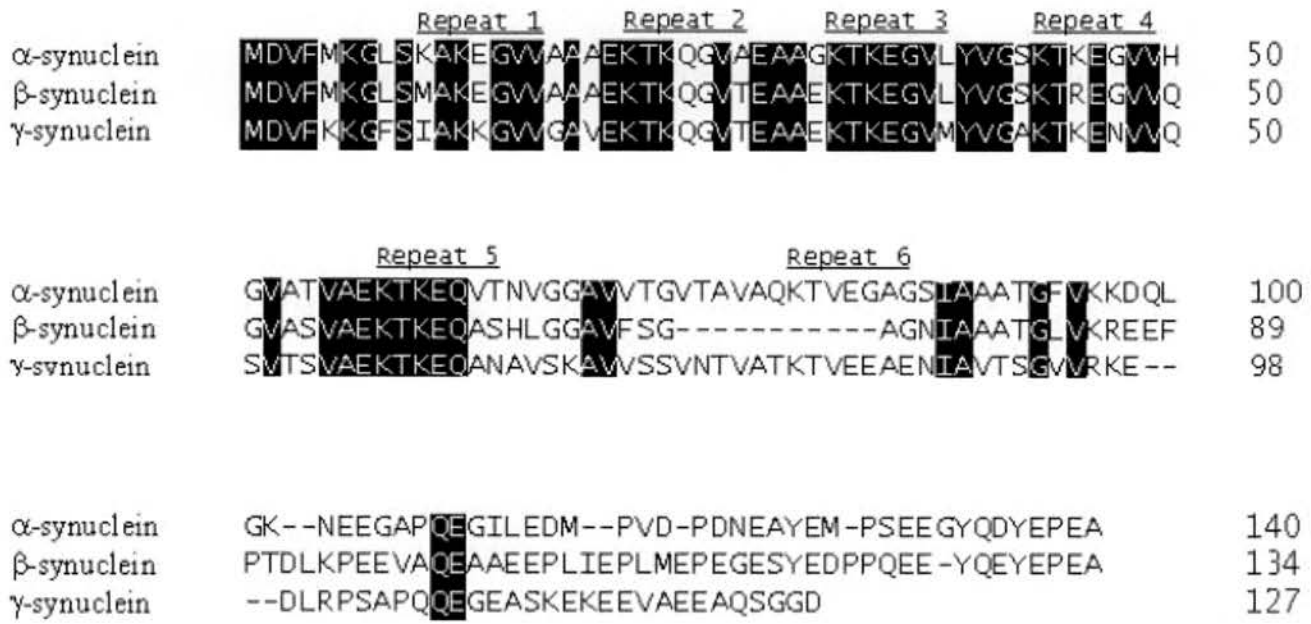
Martin, Heilig, Charbonnier, Moreau, Flaman, Petit, Hannequin, Brice and Frebourg, 1995; Chen, de Silva, Pettenati, Rao, St George-Hyslop, Roses, Xia, Horsburgh, Ueda and Saitoh, 1995; Shibasaki, Baillie, St Clair and Brookes, 1995). The 5'-untranslated region of the α-synuclein gene contains an exon with two alternative splice sites, which could be considered as two adjacent exons (exons 1 and 2).

All of the α-synuclein proteins described are identical between species for the first 42 amino acids. The human and rodent orthologs are 140 amino acids long in total and the avian ortholog synelfin is three residues longer. Human and rodent proteins share 95.3% homology and rat and mouse only differ in one residue at Ser 121.

The unique motif, which is common to all the synucleins is a repeating KTKEGV domain of which α-synuclein possesses six and was first described by Maroteaux (Martoeaux et al., 1988). The most recently identified synuclein mutation with a causative role in familial PD has also implicated these repeating domains in regulating fibrilisation of the protein. The mutation within one of these N-terminal repeats, E46K, has been shown to increase fibril formation *in vivo* (Greenbaum, Graves, Mishizen-Eberz, Lupoli, Lynch, Englander, Axelsen and Giasson, 2005). The fibrilisation of α-synuclein will be discussed in greater detail later (section 1.5.2).

Figure 1.1 Amino acid sequence alignment of the human synuclein proteins

The imperfect repeats of the type KTKEGV are identified. The black background highlights amino acid residues conserved between all three proteins. The sequences of α- and β-synucleins were obtained from Jakes, Spillantini and Goedert (1994) and γ-synuclein from Ji, Liu, Jia, Wang, Liu, Xiao, Joseph, Rosen and Shi (1997).


Repeat 1
Repeat 2
Repeat 3
Repeat 4
α-synuclein MDVFMKGLSKAKEGVVAAAEKTKQGVAEAAGKTKEGVLYVGSKTKEGVVH 50
β-synuclein MDVFMKGLSMAKEGVVAAAEKTKQGVTEAAEKTKEGVLYVGSKTREGVVQ 50
γ-synuclein MDVFKKGFSIAKKGVVGAVEKTKQGVTEAAEKTKEGVMYVGAKTKENVVQ 50
Repeat 5
Repeat 6
α-synuclein GVATVAEKTKEQVTNVGGAVVTGVTAVAQKTVEGAGSIAAATGFVKKDQL 100
β-synuclein GVASVAEKTKEQASHLGGAVFSG----------AGNIAAATGLVKREEF 89
γ-synuclein SVTSVAEKTKEQANAVSKAVVSSVNTVATKTVEEAENIAVTSGVVRKE-- 98
α-synuclein GK--NEEGAPQEGILEDM--PVD-PDNEAYEM-PSEEGYQDYEPEA 140
β-synuclein PTDLKPEEVAQEAAEEPLIEPLMEPEGESYEDPPQEE-YQEYEPEA 134
γ-synuclein --DLRPSAPQQEGEASKEKEEVAEEAQSGGD 127

1.1.3 β-synuclein

The protein which was to become β-synuclein was purified and described as an acidic neurospecific protein present in bovine brain lysate (Nakajo, Tsukada, Omata, Nakamura and Nakaya, 1993). This 14kDa protein termed phosphoneuroprotein (PNP-14) (Table 1.1) was initially thought to be neurospecific, but this is now known not to be the case (section 1.2.2) (Tobe, Nakajo, Tanaka, Mitoya, Omata, Nakaya, Tomita and Nakamura, 1992; Nakajo et al., 1993). Further studies localised PNP to synaptic membranes and observed that PNP made up 0.1% of total brain protein (Shibayama-Imazu, Okahashi, Omata, Nakajo, Ochiai, Nakai, Hama, Nakamura and Nakaya, 1993).

The β-synuclein gene (SNCB) has been localised to chromosome 5q35 and is made up of six exons, five of which are coding (Spillantini, Divane and Goedert, 1995; Lavedan, Leroy, Torres, Dehejia, Dutra, Buchholtz, Nussbaum and Polymeropoulos, 1998b). The 61% homology between bovine PNP14 and human α-synuclein was noted by Jakes and colleagues who classified the two as members of the synuclein family, referring to them as α and β (Jakes et al., 1994).

β-synuclein is a 134 amino acid protein and exhibits the highest degree of interspecies homology of all the synucleins possessing five of the characteristic KTKEGV repeat motifs. Mouse and rat orthologs are identical and share 97.8% homology with human β-synuclein which in turn shares 78% identity with human α-synuclein (Jakes et al., 1994).

β-synuclein has been shown to be phosphorylated *in vivo* by Ca^{2+}/calmodulin activated protein kinase II (CAMK-II) at residue Ser 118 which is conserved in all

species. The conservation of this residue is not seen in either α- or γ-synuclein (Nakajo et al., 1993).

1.1.4 γ-synuclein

The third member of the synuclein family was initially described in a high throughput screen as a protein abnormally expressed in cancerous breast tissue (Ji et al., 1997). Breast cancer specific gene-1 (BCSG-1) was shown to be largely absent in normal tissue but strongly expressed in malignant breast cancer tissue and was advocated as a potential marker gene (Ji et al., 1997). Homology with the NACP peptide found in Alzheimer's disease brains was subsequently observed (Ji et al., 1997). In 1998, human γ-synuclein was identified, characterised and localised to chromosome 10q23 (Lavedan et al., 1998b). This was confirmed with the independent characterisation and localisation of persyn, identical to γ-synuclein, to the long arm of chromosome 10 in the q23.2-23.3 region (Ninkina, Alimova-Kost, Paterson, Delaney, Cohen, Imreh, Gnuchev, Davies and Buchman, 1998). Synoretin was described as a member of the synuclein family expressed in retinal cells (Surguchov, Surgucheva, Solessio and Baehr, 1999). However this protein is identical to γ-synuclein and this is now the accepted nomenclature for these proteins (Table 1.1). The γ-synuclein gene (SNCG) is comprised of five exons, all of which include coding regions. This 127 amino acid protein shows the least amount of homology of all the synuclein proteins. The human protein shares 87.7 and 83.8% with the mouse and rat orthologs respectively and only 60% homology with human α-synuclein at the amino acid level (George, 2002). The *Torpedo* ortholog is a longer protein of 143 amino acids with an altered repeating motif TKQGVQDAAE, and the

last 35 amino acids bear very little homology to either the human or rodent molecule (George, 2002).

Table 1.1 Orthologs of the synuclein proteins

Table identifies the previous names of the synuclein orthologs and the papers in which they were first described. (#) represents sequences identified via sequence databases.

Synuclein Gene	Species	Previous Name	First Description
α	Human	NACP	Ueda et al., 1993
α	Rat		#
α	Mouse		#
α	Chicken		#
α	Canary	Synelfin	George et al., 1995
β	Human		#
β	Bovine	PNP-14	Nakajo et al., 1993
β	Rat	PNP-14	Shibayama-Imazu et al., 1993
β	Mouse		#
β	Chicken		#
γ	Human	BCSG-1, Persyn	Ji et al., 1997; Ninkina et al., 1998
γ	Rat		#
γ	Mouse	Persyn	Alimova-Kost et al., 1999
γ	Chicken	Persyn	Tiunova et al., 2000
γ	Bovine	Synoretin	Surguchov et al., 1999
γ	Electric ray	Synoretin	Surguchov et al., 1999

1.2 Localisation of synucleins

1.2.1 Distribution and intracellular localisation of α-synuclein

As previously mentioned, the initial description of α-synuclein localised it to the presynaptic compartment of the nerve terminals of the electric organ of *Torpedo Californica* (Maroteaux et al., 1988). In rodents, α-synuclein mRNA has been shown to be present in brain structures such as the cortex, *substantia nigra* (SN), the amygdala and hippocampus (Abeliovich, Schmitz, Farinas, Choi-Lundberg, Ho, Castillo, Shinsky, Verdugo, Armanini, Ryan, Hynes, Phillips, Sulzer and Rosenthal, 2000; Kholodilov, Neystat, Oo, Lo, Larsen, Sulzer and Burke, 1999; Lavedan, Leroy, Dehejia, Buchholtz, Dutra, Nussbaum and Polymeropoulos, 1998a).

As early as 1995, a study in rat brain reported co-localisation of α-synuclein with synaptophysin in the synaptic termini of the neocortex, hippocampus, olfactory bulb, striatum, thalamus and cerebellum (Iwai, Masliah, Yoshimoto, Ge, Flanagan, de Silva, Kittel and Saitoh, 1995). More recently, a study of murine brain showed the presence of α-synuclein protein in the SN, ventral tegmental area (VTA), striatum, globus pallidus, cortex with the most heterogeneous staining observed in the hippocampus, dentate gyrus and amygdala (Totterdell, Hanger and Meredith, 2004). Electron microscopy revealed that the majority of α-synuclein protein could be found at synaptic boutons in association with vesicle membranes (Totterdell et al., 2004).

In humans, the highest level of α-synuclein mRNA expression is found in the dopaminergic neurones of the SN (Solano, Miller, Augood, Young and Penney, 2000). It is also seen in the striatum, dentate granual cells, hippocampus, deep layers of the cortex and the globus pallidus (Abeliovich et al., 2000). An expression study of all three synucleins during the development of the SN revealed that expression

began at 15, 17 and 18 weeks gestation for α-, β- and γ-synuclein respectively, coinciding with proteins such as synaptotagmin and synaptobrevin (Galvin, Schuck, Lee and Trojanowski, 2001b). A sequential re-distribution from the perikarya of SN neurones to the nerve terminals was observed (Galvin et al., 2001b). Synaptotagmin and α-synuclein are redistributed after 18 weeks gestation, synaptobrevin and β-synuclein between 20-28 weeks and synaptophysin and γ-synuclein between 33 weeks gestation and 9 months post partum (Galvin et al., 2001b). This evidence supports the hypothesis that the synucleins have a role in the development and maturation of neurones. A study of α-synuclein expression in the mouse brain confirmed this shift in compartmentalisation (Hsu, Mallory, Xia, Veinbergs, Hashimoto, Yoshimoto, Thal, Saitoh and Masliah, 1998). The same study also confirmed that protein expression began during embryonic development and rose sharply from E12 (Hsu et al., 1998).

1.2.2 Distribution and intracellular localisation of β-synuclein

The first expression study of β-synuclein carried out in the rat concluded that protein expression began post partum and increased to a maximum level 21-28 days after birth (Shibayama-Imazu et al., 1993). β-synuclein was localised to the termini of cerebellar neurones and could also be observed in cultured midbrain neurones (Shibayama-Imazu et al., 1993). In 1994, a study of mRNA expression in adult rat brain identified β-synuclein transcripts in the granular, molecular and Purkinje layers of the cerebellar cortex, in hippocampus, striatum and cerebral cortex particularly the terminals of layer V (Nakajo, Shioda, Nakai and Nakaya, 1994). β-synuclein was reported in non-neuronal tissue in 1998 with the finding that it was associated with

fibrillar structures in Sertoli cells of the rat testes (Shibayama-Imazu, Ogane, Hasegawa, Nakajo, Shioda, Ochiai, Nakai and Nakaya, 1998).

A study of β-synuclein protein expression in the central nervous system of adult rats confirmed the pattern of mRNA expression, with positive immunostaining throughout the brain (Li, Henning Jensen and Dahlstrom, 2002). Expression was noted particularly at synaptic terminals in numerous structures including the olfactory bulb, cortex, structures of the basal ganglia, basal forebrain and amygdala (Li et al., 2002). The hippocampus, in particular the dentate gyrus, and many midbrain structures including motor nuclei such as the trocular and occularmotor nucleus also showed expression. As mentioned above, β-synuclein is expressed in the SN during gestation in humans and undergoes redistribution between 20-28 weeks from perikarya to terminals (Galvin et al., 2001b). A broad description of β-synuclein expression would be that it is widely distributed throughout the brain particularly in the cortex and in the synaptic terminals of cholinergic neurones (Li et al., 2002).

1.2.3 Distribution and intracellular localisation of γ-synuclein

γ-synuclein mRNA is expressed in the brain and spinal cord of vertebrate species, but it is most abundant in the peripheral nervous system (PNS) including neurones of the dorsal root ganglia and trigeminal ganglia beginning at E11 in the mouse embryo (Buchman, Hunter, Pinon, Thompson, Privalova, Ninkina and Davies, 1998; Tiunova, Anokhin, Saha, Schmidt, Hanger, Anderton, Davies, Ninkina and Buchman, 2000). In addition, γ-synuclein is highly expressed in the stratum granulosum of the epidermis from P5 in the mouse (Ninkina, Privalova, Pinon,

Davies and Buchman, 1999). Interestingly, γ-synuclein expression is up-regulated in advanced infiltrating breast carcinoma and over-expression in breast cancer cells augments cell motility, invasiveness and metastasis, which will be described in greater detail below (section 1.7) (Ji et al., 1997; Bruening, Giasson, Klein-Szanto, Lee, Trojanowski and Godwin, 2000).

γ-synuclein was reported as being highly expressed in the SN and in human embryos expression begins at 18 weeks gestation (Galvin, Lee and Trojanowski, 2001a). As mentioned, a translocation occurs at 33 weeks from the perikarya into the dopaminergic terminals in parallel with synaptophysin (Lavedan et al., 1998a; Galvin et al., 2001a).

A study of protein localisation in adult rat brain made the observation that γ-synuclein was expressed in fewer structures than either α- or β-synuclein and significantly that expression was seen in the perikarya of cells in many structures (Li et al., 2002). In contrast to α- and β-synuclein expression, γ-synuclein was sparsely expressed in the olfactory bulb and absent in the cortex, caudate, putamen, hippocampus and thalamus (Li et al., 2002). High levels of immunostaining were observed in the perikarya and processes of hypothalamic structures and the SN (Li et al., 2002). In addition, cells of the mammillary, red, raphe, trochlear, oculormotor, trigeminal motor and hypoglossal nuclei were seen to express γ-synuclein (Li et al., 2002). Moving caudally, the pons, the nerve terminals of the locus coreleus displayed intense γ-synuclein staining as did Lamina I and II of the spinal cord (Li et al., 2002). In summary, expression of γ-synuclein appeared very weak or was virtually absent from the forebrain. However, it was abundant in somatic cholinergic nuclei and in

autonomic components in the basal forebrain, the brainstem and the spinal cord (Li et al., 2002).

The significance of the differential distribution of α-, β- and γ-synucleins is not clear. However, these differences could imply functional differences between these proteins.

1.3 Physiological function of the synucleins

1.3.1 Functional characteristics of synucleins

The hypothesis that the synucleins have a role in synaptic plasticity was supported by increased expression of the α-synuclein avian ortholog synelfin during a critical patterning phase of neuronal plasticity in the development of the zebra finch (George et al., 1995). Subsequent studies support the idea that synucleins are important in the maturation of a developing synapse once synaptic connections have formed. For example, in cultured rat hippocampal neurones α-synuclein is not detected until five days after connections formed in culture (Withers, George, Banker and Clayton, 1997).

There is no doubt that the synucleins are present at the presynaptic terminal. What is in doubt is their precise role. Synucleins may be exclusively associated with synaptic vesicles or they may interact with other parts of synaptic infrastructure binding only transiently to lipids. Interestingly, α-synuclein is not enriched in purified synaptic vesicle preparations (George et al., 1995; Irizarry, Kim, McNamara, Tanzi, George, Clayton and Hyman, 1996), which suggests that synucleins does not bind directly to vesicles. However, other mutations demonstrated that α-synuclein could interact directly with lipid vesicles (Davidson, Jonas, Clayton and George, 1998).

The protein structure of α-synuclein suggests the presence of amphipathic alpha-helical domains, similar to the exchangeable apolipoproteins (Davidson et al., 1998). It was hypothesised by Davidson and colleagues that α-synuclein should associate with phospholipid bilayers and that this lipid association should stabilise the α-helical secondary structure in the protein (Davidson et al., 1998). α-synuclein binds small unilamellar vesicles containing acidic phospholipids, but not to vesicles with a net neutral charge (Davidson et al., 1998). Lipid binding was accompanied by an increase in α-helicity from 3% to approximately 80% (Davidson et al., 1998). Subsequently, the exact nature of this interaction has been characterised in greater detail and α-synuclein has been shown to interact with acidic vesicles via the N-terminal (Ramakrishnan, Jensen and Marsh, 2003). A conformational change occurs as the hydrophobic N-terminal interacts with the vesicle changing from a flattened conformation into two α-helical domains separated by a two amino acid break (Chandra, Chen, Rizo, Jahn and Sudhof, 2003). From these results it was hypothesised that α-synuclein forms a surface-active coat for phospholipid membranes at the presynaptic membrane. The N-terminal homology shared between the three synucleins suggests that all the proteins would form the same structures (Chandra et al., 2003). However, α-synuclein differs from the other two synucleins as it has a longer second α-helix than β-synuclein, and has a more hydrophobic surface than γ-synuclein (Chandra et al., 2003). This difference may explain the selective propensity of α-synuclein to aggregate and thus explain its involvement in neurodegenerative diseases.

A loss of α-synuclein expression has been shown to precipitate a reduction in the number of reserve pool vesicles in hippocampal neurones both *in vitro* (Murphy,

Rueter, Trojanowski and Lee, 2000) and *in vivo* (Cabin, Shimazu, Murphy, Cole, Gottschalk, McIlwain, Orrison, Chen, Ellis, Paylor, Lu and Nussbaum, 2002). However, there is little effect on vesicles docked at the presynaptic membrane, implying a role in vesicle formation possibly through an interaction with synphillin-1, a protein shown to interact with α-synuclein in yeast two-hybrid screens (Ribeiro, Carneiro, Ross, Menezes and Engelender, 2002).

1.3.2 Synucleins and the dopamine transporter

Controversy has arisen recently regarding the association of α-synuclein with the dopamine transporter (DAT). The DAT is a member of a large gene family of Na^+ and Cl^- dependent transporters. They share a common topology of 12 putative transmembrane (TM) domains, intracellular amino and carboxyl termini, a large extracellular loop between TM 3 and 4 which contain numerous consensus sequences for *N*-linked glycosylation (Loland, Norgaard-Nielsen and Gether, 2003). There are putative sites for phosphorylation by protein kinase A (PKA), protein kinase C (PKC) and CAMK-II within intracellular domains including the N- and C- termini (Reith and Chen, 1997). The DAT is expressed in presynaptic terminals of SN neurones (Reith and Chen, 1997).

Initial results indicated that α-synuclein complexed directly with DAT via the C-terminal tail of the transporter in human neurones and co-transfected cells (Lee, Liu, Pristupa and Niznik, 2001). This complex promotes clustering of the DAT in the membrane thereby accelerating the re-uptake of dopamine from the synaptic cleft possibly promoting excitotoxicity, which will be discussed later (section 1.5.3)

(Reith and Chen, 1997; Lee et al., 2001). These results would implicate α-synuclein directly in a possible cytotoxic mechanism.

However, other studies have noted the formation of a stable complex between α-synuclein and DAT, with directly contradictory results. Wersinger et al. (2003) reported an inhibitory effect on dopamine re-uptake in co-transfected cells, with a reduced rate of flow as a function of α-synuclein concentration (Wersinger, Prou, Vernier, Niznik and Sidhu, 2003). *In vivo* studies carried out to compare α-synuclein null mutant mice to wild-types have revealed that the absence of α-synuclein has no effect on the kinetics of DAT (Dauer, Kholodilov, Vila, Trillat, Goodchild, Larsen, Staal, Tieu, Schmitz, Yuan, Rocha, Jackson-Lewis, Hersch, Sulzer, Przedborski, Burke and Hen, 2002).

There is considerable evidence to suggest that α-synuclein does indeed form a complex with DAT. However, the purpose and effect of this association is unclear. Nonetheless the ability of α-synuclein to bind and modulate dopamine re-uptake may have the potential to impact upon neuronal survival, and thus had direct relevance to the pathological processes observed in synucleinopathies.

1.3.3 Chaperone activity of the synucleins

Many interaction partners have been reported for α-synuclein, including the extracellular signal-regulated kinases (ERK1/2; Ostrerova, Petrucelli, Farrer, Mehta, Choi, Hardy and Wolozin, 1999), 14-3-3 (Ostrerova et al., 1999), Tat binding protein 1 (Ghee, Fournier and Mallet, 2000), parkin (Shimura, Schlossmacher, Hattori, Frosch, Trockenbacher, Schneider, Mizuno, Kosik and Selkoe D, 2001), tau and microtubule associated protein 1B (Jensen, Hager, Nielsen, Hojrup, Gliemann and

Jakes, 1999), β-synuclein (Hashimoto, Rockenstein, Mante, Mallory and Masliah, 2001) Aβ and the β-amyloid peptide (NACP) (Yoshimoto, Iwai, Kang, Otero, Xia and Saitoh, 1995). α-synuclein has also been shown to modulate the activity of several crucial proteins via its interaction with them. These include tyrosine hydroxylase (TH) (Perez and Zigmond, 2000), PKC (Ostrerova et al., 1999), ERK2 (Iwata, Miura, Kanazawa, Sawada and Nukina, 2001) and phospholipase D2 (PLD2) (Jenco, Rawlingson, Daniels and Morris, 1998). The evidence showing the ability of α-synuclein to bind and in some cases modulate the activity of a wide range of different proteins has led to the hypothesis that α-synuclein is a chaperone protein (Perez and Hastings, 2004).

Biochemical evidence shows that recombinant α-synuclein has a chaperone-like function against thermal and chemical stress *in vitro*, protecting glutathione S-transferase (GST) and aldolase from heat-induced precipitation, and alpha-lactalbumin and bovine serum albumin from dithiothreitol-induced precipitation like other molecular chaperones (Kim, Paik, Yang and Kim, 2000). In addition, α-synuclein can be rapidly and significantly precipitated by heat in the presence of Zn^{2+} ions *in vitro*, whereas it was not affected by the presence of Ca^{2+} or Mg^{2+} ions (Kim et al., 2000). Circular dichroism spectra confirmed that α-synuclein undergoes a conformational change in the presence of Zn^{2+} (Kim et al., 2000), possibly to regulate its chaperone activity.

14-3-3 proteins are a family of chaperone proteins which are also widely expressed in the brain (Layfield, Fergusson, Aitken, Lowe, Landon and Mayer, 1996). α-synuclein shares up to 40% sequence homology with 14-3-3 proteins and can bind to 14-3-3 proteins providing yet more evidence of a role for α-synuclein as a chaperone

(Ostrerova et al., 1999). Another significant finding linking α-synuclein with 14-3-3 proteins is their shared ability to bind TH, the critical rate-limiting enzyme involved in the dopamine synthesis pathway (Ichimura, Isobe, Okuyama, Takahashi, Araki, Kuwano and Takahashi, 1988; Perez and Zigmond, 2000). α-synuclein is a negative regulator of TH *in vitro*, thereby inhibiting normal function, precipitated as a reduction in dopamine synthesis in transfected dopaminergic cells (Perez, Waymire, Lin, Liu, Guo and Zigmond, 2002).

The interactions of α-synuclein with TH and DAT, combined with its ability to affect synaptic vesicles, may thus have specific implications for nigral dopaminergic neurones during the pathogenesis of Parkinson's disease. Chaperone activity is not restricted to α-synuclein; β- and γ-synuclein have also displayed this activity (Souza, Giasson, Lee and Ischiropoulos, 2000). All three synucleins have been shown to suppress the aggregation of thermally denatured alcohol dehydrogenase and chemically denatured insulin (Souza et al., 2000). This same study also revealed that residues 98-140 within the C-terminal are critical to the chaperone activity of α-synuclein as this activity is lost upon removal of this stretch of amino acids. The C-terminal is a highly acidic region which displays the least amount of homology between the synuclein proteins, suggesting this region may be the source of the specificity and activity of individual synucleins.

Subsequent studies of α-synuclein revealed that the N-terminal domain (residues 1-95) bind to substrate proteins to form high molecular weight complexes. The C-terminal acidic tail (residues 96-140) appears to be primarily involved in solubilising the high molecular weight complexes. This suggests that it is the C-terminal domain which is responsible for the specificity of the chaperone activity (Park, Jung, Kim,

Park, Yang and Kim, 2002; Lee, Paik and Choi, 2004). β-synuclein has recently been shown to be a more effective chaperone than α-synuclein when suppressing the heat activated aggregation of adolase, alcohol dehydrogenase and citrate synthase (Lee et al., 2004). Moreover, β-synuclein inhibits the amyloid formation of both Aβ and α-synuclein, suggesting a protective role *in vivo* (Lee et al., 2004).

1.3.4 Regulatory effects of synucleins

An animal model with an α-synuclein null mutation showed enhanced striatal dopamine (DA) release when compared with wild-type mice in a paired pulse paradigm of hippocampal plasticity (Abeliovich et al., 2000). This suggested that TH may be more active in the absence of any inhibitory effects α-synuclein may have. The absence of α-synuclein would be expected to increase TH activity therefore producing more DA for release at the second pulse. Curiously, the α-synuclein null mutant had a slight but significant reduction in striatal DA levels (Abeliovich et al., 2000). This could be the result of a reduction in the efficiency of DAT in the absence of α-synuclein, but this hypothesis has not yet been tested. It is noteworthy that a different strain of α-synuclein null mutant mice generated by Thomas Sudhof and colleagues had no reduction in striatal DA (Schluter, Fornai, Alessandri, Takamori, Geppert, Jahn and Sudhof, 2003).

A second factor influenced by α-synuclein which may also have bearing on neurotransmitter levels could be a change in the number of synaptic vesicles. However, Sudhof and colleagues saw no change in vesicle proteins, implying that normal vesicle numbers were maintained throughout the brain in these α-synuclein null mice (Schluter et al., 2003). Reduced numbers of vesicles were seen in

hippocampal neurones in a third of α-synuclein null mice (Cabin et al., 2002), supporting findings of Murphy and colleagues who saw a similar reduction in vesicle number in hippocampal neurones when treated with antisense-α-synuclein oligonucleotides (Murphy et al., 2000).

The mice produced and described by Schulter et al. (2003) displayed a small but significant increase in β-synuclein expression in the striatum suggesting a compensatory mechanism in the absence of α-synuclein. This hypothesis is supported by the finding that both α- and β-synuclein can efficiently inhibit the activity of PLD2 (Jenco et al., 1998), suggesting some redundancy of function between these synuclein family members.

1.3.5 Proteosomal inhibition

Recent studies indicate that aggregated α-synuclein binds to S6', a component of the 19S in the 26S proteasome, and inhibits 26S proteasomal degradation in both a ubiquitin-independent and ubiquitin-dependent manner (Snyder, Mensah, Hsu, Hashimoto, Surgucheva, Festoff, Surguchov, Masliah, Matouschek and Wolozin, 2005). Monomeric α- and β-synuclein were shown to inhibit 20S and 26S proteosome activity weakly, while γ-synuclein strongly inhibited ubiquitin-independent proteolysis (Snyder et al., 2005). Aggregated α-synuclein molecules were shown to have an inhibitory effect on ubiquitin-independent proteosomal degradation (Snyder et al., 2005). Co-incubating these proteins in various combinations revealed that while β-synuclein has no direct effect on proteosomal degradation it does act as a negative regulator of α-synuclein (Snyder et al., 2005).

1.4 The pathological significance of the synucleins

1.4.1 Synucleinopathies

Synucleinopathies, or α-synucleinopathies as they are sometimes termed, are a collection of neurodegenerative disorders that share one common feature; cytoplasmic inclusions of which α-synuclein is a constituent part, as described below (Galvin, Uryu, Lee and Trojanowski, 1999). However, this definition is not precisely accurate as the other synucleins have been detected in lesions of certain neurodegenerative diseases (Galvin, Giasson, Hurtig, Lee and Trojanowski, 2000). The following is a summary of these "synucleinopathies" and the role the synucleins play in their pathology.

1.4.2 Alzheimer's disease

The link between neurodegenerative disease and α-synuclein was first reported for Alzheimer's disease (AD) when the small peptide termed "NAC" was purified from an AD brain, leading to the cloning of the non-Aβ component precursor protein (NACP) (Ueda et al., 1993) now known to be a fragment of α-synuclein. It was thought that NACP, a 35 amino acid fragment of α-synuclein, was a constituent of the AD senile plaques, the extracellular lesions abundant in AD brain (Ueda et al., 1993). However, recent evidence revealed that α-synuclein was not an integral component of senile plaques in either human AD (Culvenor, McLean, Cutt, Campbell, Maher, Jakala, Hartmann, Beyreuther, Masters and Li, 1999) or in transgenic mouse models of AD, but was sometimes present in neurites at the plaque periphery (Yang, Ueda, Chen, Ashe and Cole, 2000).

1.4.3 Parkinson's disease

Parkinson's disease (PD) is a progressive neurodegenerative disorder that is becoming more prevalent with a decreasing age of onset, currently afflicting around 1.6% of Europe's over sixty five population, four million globally (World Health Organisation, 1998). The condition was first described in the 1817 monograph, "*Essay on the shaking palsy*" by James Parkinson. It is characterised by severe motor symptoms including uncontrollable tremor, postural imbalance, slowness of movement and rigidity. The pathological hallmarks of this disorder are the presence of eosinophilic inclusions named Lewy bodies (LB) (section 1.5.1) and a pronounced loss of dopaminergic neurones in the *substantia nigra pars compacta* (SNpc). This neuronal depletion results in a reduction in the levels of striatal dopamine, thus disrupting the feedback regulation in the basal ganglia and hence motor function. LBs, which contain α-synuclein are thought to be neurotoxic and hence play a role in neurodegeneration.

1.4.4 Etiological factors of Parkinson's disease

The disease can arise sporadically, as a result of environmental factors or can be due to genetic factors, discussed below. Environmental toxins, including transition metals such as iron and copper, have been identified as potential risk factors (Gorell, Johnson, Rybicki, Peterson, Kortsha, Brown and Richardson, 1997). Accumulation of these metal ions within neurones is thought to be toxic as the metals participate in oxidation reactions leading to neurotoxic products (Dexter, Wells, Lees, Agid, Agid, Jenner and Marsden, 1989). Metals have also been shown to interact with α-synuclein to promote fibrilisation (Uversky et al., 2001). Pesticides are another class

of agent with an apparent neurotoxic effect relevant to PD. Agricultural workers exposed to pesticides have been shown to have increased risks of developing PD in a dose-dependant manner (Semchuk, Love and Lee, 1992; Gorell, Johnson, Rybicki, Peterson and Richardson, 1998). Chemicals such as bipyridyl (paraquat), organochlorine and carbamate are all widely used and have been implicated in PD (Semchuk et al., 1992).

1.4.5 The genetic link to Parkinson's disease

Sporadic forms of the disease account for 95% of cases. Nevertheless, studying rare genetic forms of the disease may uncover phenotypic similarities between genetic and sporadic forms. This would indicate a common pathogenic mechanism such that genetic analysis might reveal a common key biochemical pathway to target for therapeutic development. This is particularly significant here as PD is the only synucleinopathy in which a genetic mutation of the α-synuclein gene has been directly linked with pathogenesis.

Five PD genes have been identified and studied in some detail; α-synuclein, parkin, ubiquitin C-terminal hydrolase L1 (UCHL-1), DJ-1 and PINK1. All have been shown to participate in the ubiquitin-proteasome pathway, a particularly compelling finding considering the Lewy body (LB) protein aggregates that characterise PD neuropathology.

1.4.6 α-synuclein and Parkinson's disease

Interest in synucleins accelerated in the late 90's with the discovery of two point mutations, A53T and A30P at the α-synuclein locus, present in families suffering

early onset PD (Polymeropoulos et al., 1997; Kruger et al., 1998). The A53T translocation in α-synuclein was identified in a number of Italian kindred and three unrelated Greek families found on the long arm of human chromosome 4 (Polymeropoulos et al., 1997). The phenotypic appearance of the symptoms due to this mutation were typical for PD, including the presence of Lewy bodies (section 1.5.1), the only exception being the relatively early age of onset, 46 ± 13 years (Polymeropoulos et al., 1997). Within the large Italian family, the penetrance of the mutated gene was approximately 85% suggesting that this single genetic defect was sufficient to determine the PD phenotype (Polymeropoulos et al., 1997). The A30P mutation has only been identified in one German family with autosomal dominantly-inherited parkinsonism (Kruger et al., 1998).

The fact that fibrillated α-synuclein is abundant in LBs suggests that its propensity to misfold and form amyloid fibrils may be responsible for its neurotoxicity in pathological conditions such as PD and that pathogenic mutations precipitate a toxic function (Wakabayashi, Engelender, Yoshimoto, Tsuji, Ross, Takahashi, 2000). Misfolding of α-synuclein may interfere with its normal functions, but it is unlikely that loss of function plays a major role in α-synuclein-related neurodegeneration (Abeliovich et al., 2000; Dauer et al., 2002). Both wild-type and mutant α-synuclein form amyloid fibrils resembling those seen in LBs (Conway, Harper and Lansbury, 1998; Giasson, Uryu, Trojanowski and Lee, 1999) and nonfibrillary oligomers termed "protofibrils" (Conway et al., 1998). The two known pathogenic α-synuclein mutations (A53T + A30P) promote the formation of protofibrils, suggesting they may be the toxic species of α-synuclein (Conway, Lee, Rochet, Ding, Harper, Williamson and Lansbury, 2000). Consistent with this view and the association of α-

synuclein with synaptic vesicles, protofibrils may cause toxicity by permeabilising synaptic vesicles (Volles, Lee, Rochet, Shtilerman, Ding, Kessler and Lansbury, 2001; Lashuel, Petre, Wall, Simon, Nowak, Walz and Lansbury, 2002), allowing DA to leak into the cytoplasm and participate in reactions that generate oxidative stress as discussed below (section 1.5.3). To date protofibrils have only been observed and studied *in vitro*, so further work will need to explore whether they form in neurones and if their formation correlates with neurotoxicity.

In addition to the original two α-synuclein point mutations which linked synucleins with familial PD, a third mutation has been shown to cause PD within a group known as the Iowa kindred (Singleton, Farrer, Johnson, Singleton, Hague, Kachergus, Hulihan, Peuralinna, Dutra, Nussbaum, Lincoln, Crawley, Hanson, Maraganore, Adler, Cookson, Muenter, Baptista, Miller, Blancato, Hardy and Gwinn-Hardy, 2003). This mutation is a triplication of the α-synuclein gene at the correct locus on one chromosome resulting in overproduction and accumulation of α-synuclein. Excess cytosolic α-synuclein has shown to increase the likelihood of fibril formation and hence increased neurotoxicity (section 1.5.2).

The most recent α-synuclein mutation linked to neurodegenerative conditions is the E46K point mutation within one of the characteristic KTKEGV N-terminal repeat regions. This was identified within a Spanish family exhibiting Parkinson's disease and Lewy body dementia (Zarranz, Alegre, Gomez-Esteban, Lezcano, Ros, Ampuero, Vidal, Hoenicka, Rodriguez, Atares, Llorens, Gomez Tortosa, del Ser, Munoz and de Yebenes, 2004). This fostered the hypothesis that these repeats, specifically glutamate residues, may regulate α-synuclein fibrilisation. This was found to be the case by Greenbaum et al, who also reported that point mutations

replacing other glutamate residues within other repeats had the same effect (Greenbaum et al., 2005).

1.4.7 Parkin

Parkin is a second gene identified as a cause of familial PD. Although this form was originally termed autosomal recessive juvenile parkinsonism, the clinical phenotype is now known to include older-onset patients (Kitada, Asakawa, Hattori, Matsumine, Yamamura, Minoshima, Yokochi, Mizuno and Shimizu, 1998; Lincoln, Wiley, Lynch, Langston, Chen, Lang, Rogaeva, Sa, Munhoz, Harris, Marder, Klein, Bisceglio, Hussey, West, Hulihan, Hardy and Farrer, 2003). In general, however, parkin mutations are found in PD patients with onset before age 30, particularly those with a family history consistent with recessive inheritance (Mizuno, Hattori, Kitada, Matsumine, Mori, Shimura, Kubo, Kobayashi, Asakawa, Minoshima and Shimizu, 2001). Unlike the PD arising from mutations in α-synuclein, Lewy Bodies are not seen in the parkinsonism triggered by Parkin mutations (Mizuno, Hattori and Matsumine, 1998). However the fundamental pathology remains the same in either case, i.e. the irreversible loss of dopaminergic neurones in the SNpc. It is uncertain how loss of parkin function leads to dopaminergic neurone degeneration, but clues are emerging from the identification of its physiological function. Parkin is an E3 ubiquitin ligase (Zhang, Gao, Chung, Huang, Dawson and Dawson, 2000; Shimura et al., 2001), a component of the ubiquitin-proteasome system that identifies and targets misfolded proteins to the proteasome for degradation (Sherman and Goldberg, 2001). The upstream ubiquitin ligases (E1 and E2) co-operate non-specifically to tag misfolded proteins with a single ubiquitin protein, while E3 ligases confer target

specificity by binding to specific molecules or classes of molecules facilitating the polyubiquitination necessary for targeting to the proteasome. Many parkin mutations abolish this E3 ligase activity, suggesting that the accumulation of misfolded parkin substrates could be responsible for the demise of SNpc dopaminergic neurones in PD (Petrucelli, O'Farrell, Lockhart, Baptista, Kehoe, Vink, Choi, Wolozin, Farrer, Hardy and Cookson, 2002).

Three reports suggest a relationship between parkin and synuclein function or aggregation (Shimura et al., 2001; Petrucelli et al., 2002; Chung, Zhang, Lim, Tanaka, Huang, Gao, Ross, Dawson and Dawson, 2001). Notably, the E3 ligase activity of parkin modulates the sensitivity of cells to both proteasome inhibitor- and mutant synuclein-dependent cell death (Petrucelli et al., 2002). A number of observations suggest that the functional interaction between α-synuclein and parkin may involve the proteasome. Synuclein interacts with and is possibly degraded by the proteasome (Ghee et al., 2000; Snyder, Mensah, Theisler, Lee, Matouschek and Wolozin, 2003), synuclein over-expression can inhibit the proteasome (Stefanis, Kholodilov, Rideout, Burke and Greene, 2001) and mutant synuclein increases the sensitivity of cells to proteasome inhibition (Tanaka, Engelender, Igarashi, Rao, Wanner, Tanzi, Sawa, Dawson, Dawson and Ross, 2001; Petrucelli et al., 2002).

1.4.8 Other genetic triggers of Parkinson's disease

Three other genetic triggers for Parkinson's disease have been identified but are not currently known to involve α-synuclein directly. A dominant mutation (I93M) in UCH-L1 was identified in one family with inherited PD but no pathological data were included in this report (Leroy, Boyer, Auburger, Leube, Ulm, Mezey, Harta,

Brownstein, Jonnalagada, Chernova, Dehejia, Lavedan, Gasser, Steinbach, Wilkinson and Polymeropoulos, 1998). The UCH-L1 enzyme catalyses the hydrolysis of C-terminal ubiquityl esters and is thought to play a role in recycling ubiquitin ligated to misfolded proteins after their degradation by the proteasome (Wilkinson, 2002). Although the I93M mutation decreases the activity of this uquitinating enzyme, UCH-L1 null mice do not display dopaminergic neurodegeneration (Saigoh, Wang, Suh, Yamanishi, Sakai, Kiyosawa, Harada, Ichihara, Wakana, Kikuchi and Wada, 1999). Rather, they develop an axonopathy that affects sensory axons in the gracile nucleus of the medulla (Saigoh et al., 1999). A second mutation S18Y has been shown to have the converse effect by promoting ligase activity, thus acting in a neuroprotective manner (Liu, Fallon, Lashuel, Liu and Lansbury, 2002).

DJ-1 is a homodimeric multifunctional protein ubiquitously expressed throughout human tissues (Bonifati, Dekker, Vanacore, Fabbrini, Squitieri, Marconi, Antonini, Brustenghi, Dalla Libera, De Mari, Stocchi, Montagna, Gallai, Rizzu, van Swieten, Oostra, van Duijn, Meco and Heutink, 2002). Eleven different DJ-1 mutations, missense, truncation, splice site and large deletions have been linked to autosomal recessive forms of PD (Bonifati et al., 2002; Hague, Rogaeva, Hernandez, Gulick, Singleton, Hanson, Johnson, Weiser, Gallardo, Ravina, Gwinn-Hardy, Crawley, St George-Hyslop, Lang, Heutink, Bonifati, Hardy and Singleton, 2003). One family carried a deletion predicted to abolish protein function, while the other harboured a missense mutation that results in the insertion of a proline into an α-helical region (Hague et al., 2003). Expression of this proline mutant form of DJ-1 appears to lead to its accumulation in mitochondria (Bonifati et al., 2002), and DJ-1 has been

implicated as a cellular monitor of oxidative stress (Mitsumoto and Nakagawa, 2001; Mitsumoto, Nakagawa, Takeuchi, Okawa, Iwamatsu and Takanezawa, 2001). The cytotoxic implications of oxidative stress are discussed in greater detail below (section 1.5.3). As this discovery is recent, no pathological studies have been performed hence it is not known if typical parkinsonian hallmarks are present.

A Sicilian family has been identified which carries a mutation at the PARK 6 locus, 1p35-36, associated with an autosomal recessive form of early onset PD (Valente, Bentivoglio, Dixon, Ferraris, Ialongo, Frontali, Albanese and Wood, 2001). Subsequently, a further eight families have been identified as carrying mutations in this region, sequencing revealed two homozygous mutations in the gene encoding PTEN-induced putative kinase-1 (PINK1) (Valente, Salvi, Ialongo, Marongiu, Elia, Caputo, Romito, Albanese, Dallapiccola and Bentivoglio, 2004). PINK1 is a 581 amino acid protein expressed ubiquitously in mouse and at higher levels in the brain (Valente et al., 2004). A single serine/threonine kinase domain appears to be the only functional domain, however a mitochondrial trafficking signal may be present as the protein was found to localise there in transfected cell lines (Valente et al., 2004). Although critical substrates have not yet been identified, loss of correct function appears to be sufficient to trigger PD.

1.4.9 Multiple system atrophy

Multiple system atrophy (MSA) is a sporadic neurodegenerative disease of unknown etiology with adult onset. The characteristic symptoms of MSA include parkinsonism, ataxia and autonomic failure in any combination. The term MSA was first coined in 1969 by Graham and Oppenheimer to describe a group of patients

displaying symptoms of an unknown disorder which affected the pyramidal, extrapyramidal, cerebellar and autonomic pathways (Graham and Oppenheimer, 1969). This term encompassed disorders previously named striatonigral degeneration (SND), sporadic olivopontocerebellar atrophy (OPAC) and the Shy-Drager Syndrome (SDS). Subsequently, further distinctions have been made. Patients with predominantly parkinsonian symptoms were designated MSA-P rather than striatonigral degeneration and those with predominantly cerebellar symptoms, MSA-C as opposed to olivopontcerebellar atrophy (Kato, Nakamura, Hirano, Ito, Llena and Yen, 1991; Papp and Lantos, 1992). The defining evidence that MSA was a clinico-pathological entity was the discovery of argyrophillic glial cytoplasmic inclusions (GCI's) in the oligodendrocytes of patients with phenotypically varied MSA (Papp and Lantos, 1992). Similar inclusions were identified in glial nuclei (GNI's), neuronal nuclei (NNI's), neuronal cytoplasm (NCI's) and also in neuronal axons (NAI's) (Papp and Lantos, 1992). The mechanism behind the formation of these inclusion bodies is still unclear, as is the nature of their cytotoxicity. However, the discovery of α-synuclein as part of these inclusions begins to shed light on some of the symptomatic and pathological similarities with PD and Lewy bodies, leading to the inclusion of MSA as a synucleinopathy. α-synuclein is now known to be a major component of GCI's, NCI's and NNI's but not of GNI's (Goedert and Spillantini, 1998; Gai, Power, Blumbergs and Blessing, 1998; Tu, Galvin, Baba, Giasson, Tomita, Leight, Nakajo, Iwatsubo, Trojanowski and Lee, 1998).

In fixed MSA brain sections, GCIs were composed of amorphous material-coated filaments up to 30 nm in size (Gai, Pountney, Power, Li, Culvenor, McLean, Jensen and Blumbergs, 2003). The filaments were often organised in parallel bundles

extending into oligodendroglial processes. In freshly isolated GCIs, progressive buffer washes removed amorphous material and revealed that GCI filaments consisted of 10nm sized central core fibrils that were strongly α-synuclein immunoreactive (Gai et al., 2003). Analysis revealed that each of these core fibrils was made of two subfibrils, and each of which was made of a string of 3 to 6nm sized particles probably α-synuclein oligomers (Gai et al., 2003). Immunogold labelling demonstrated that epitopes encompassing entire α-synuclein molecules were represented in the core fibrils, with the N-terminal 11–26 and C-terminal 108–131 amino acid residues most accessible to antibodies, probably exposed on the surface of the fibril (Gai et al., 2003). This study indicated that GCI filaments are multilayered in structure, with α-synuclein oligomers forming the central core fibrils of the filaments.

1.4.10 Dementia with Lewy bodies

Dementia with Lewy bodies (DLB) is now considered the second most common cause of dementia accounting for 20% of recorded cases, behind Alzheimer's which accounts for 60% (McKeith, 2004). DLB tends to affect more males than females with the age of onset falling between 50 and 80 (Cercy and Bylsma, 1997). The three core diagnostic features that must be present in order for a diagnosis of DLB to be given are fluctuating alertness/cognition, fully formed visual hallucinations and spontaneous Parkinson's disease (Ballard, McKeith, Harrison, O'Brien, Thompson, Lowery, Perry and Ince, 1997; McKeith, 2004). REM sleep behaviour disorder (RBD) often accompanies DLB and is a useful marker when distinguishing DLB

from AD (Boeve, Silber, Ferman, Kokmen, Smith, Ivnik, Parisi, Olson and Petersen, 1998).

The dementia in DLB takes the form of a progressive cognitive deterioration accompanied by a general loss of functional ability. Initial complaints include a short/variable attention span and visual perceptual difficulties, not always a primary memory complaint. The first case was described in 1961 (Okazaki, Lipkin and Aronson, 1961). In PD, Lewy body formation occurs predominantly in the SN. In DLB, formation occurs predominantly in the SN and locus coerulus but also cerebral cortex, particularly in cingular gyrus, the parahippocampal gyrus, the amygdala, the temporal neocortex and in the nuclei of the brain stem (McKeith, 2004).

An extensive depletion of cholinergic neurotransmission occurs in neocortical areas as a direct result of neurodegeneration in areas of the brainstem and basal forebrain. Neurodendritic degeneration also occurs within certain populations as a direct result of the formation of Lewy neurites that stain positive for α-synuclein and ubiquitin (Fig. 1.2a) (Arai, Ueda, Ikeda, Akiyama, Haga, Kondo, Kuroki, Niizato, Iritani and Tsuchiya, 1998; Irizarry, Growdon, Gomez-Isla, Newell, George, Clayton and Hyman, 1998; Kruger and Schulz, 2002). Such degeneration is seen in the SN, hippocampal regions, dorsal vagal nuclei, basal nucleus of Meynert and the transentorhinal cortex (Gomez-Tortosa, Newell, Irizarry, Albert, Growdon and Hyman, 1999). Like most other neurodegenerative conditions there is no absolute cure, however acetylcholine-esterase inhibitors can ease neuropsychotic symptoms by attenuating cholinergic depletion (Simard and van Reekum, 2004).

1.4.11 Pantothenate kinase-associated neurodegeneration (Hallervorden-Spatz syndrome)

Hallervorden-Spatz syndrome (HSS) involves neurodegeneration with brain iron accumulation type-1 (NBIA-1) and is a rare inherited autosomal recessive neurological movement disorder characterised by progressive degeneration of the nervous system, first described in 1922 (Hallervorden and Spatz, 1922). Recently, the pantothenate kinase gene (PANK2) has been identified as the causative gene for HSS (Zhou, Westaway, Levinson, Johnson, Gitschier and Hayflick, 2001). The detection of several mutations in almost all exons of PANK2 has led to the renaming of HSS to pantothenate kinase-associated neurodegeneration (PKAN). Symptoms, which vary greatly among patients and usually develop during childhood, are unique among the synucleinopathies and may include slow writhing, distorting muscle contractions of the limbs, face or trunk, choreoathetosis (involuntary, purposeless jerky muscle movements), muscle rigidity (uncontrolled tightness of the muscles), spasticity (sudden, involuntary muscle spasms), ataxia (inability to coordinate movements), confusion, disorientation, seizures, stupor and dementia (Vasconcelos, Harter, Duffy, McDonough, Seidman and Seidman, 2003). Other less common symptoms may include painful muscle spasms, dysphasia (difficulty speaking), mental retardation, facial grimacing, dysarthria (poorly articulated speech) and visual impairment (Vasconcelos et al., 2003).

Pathological investigations of PKAN brains have revealed mild fronto-temporal atrophy and discoloration of the globus pallidus and the SNpr (Neumann, Adler, Schluter, Kremmer, Benecke and Kretzschmar, 2000). Typical histological features include widespread axonal spheroids and large deposits of iron pigment in the

discoloured regions and excessive numbers of Lewy bodies (LBs) throughout the brain stem and cortical regions (Neumann et al., 2000). LBs as well as Lewy neurites in cases of PKAN, were strongly labelled by antibodies against α-synuclein (Neumann et al., 2000; Galvin et al., 2000). Other spheroidal inclusions which do not resemble LBs have been identified and significantly the presence of β- and γ-synuclein has been detected implicating all three synuclein proteins in the pathology of the disease (Galvin et al., 2000).

It is clear that the aberrant expression and/or changes in intracellular compartmentalisation of synucleins has dire consequences in the nervous system. To make progress in understanding the pathology of these conditions, the physiological function of the synucleins must be elucidated.

1.5 Pathogenic α-synuclein

1.5.1 Lewy bodies

Lewy bodies (LBs) are eosinophilic inclusions found in the cell bodies and neurites of certain neuronal populations, most notably the SNpc. LBs are present in normal aging brains, but are also the pathological hallmark of Parkinson's disease as well as being present in other synucleinopathies such as dementia with Lewy bodies (DLB) (Forster and Lewy, 1912; Goedert, Spillantini, Serpell, Berriman, Smith, Jakes and Crowther, 2001).

Significantly, LBs (Fig. 1.2b) contain α-synuclein filaments, 20-600nm in length and 5-10nm in diameter and the α-synuclein binding protein synphilin-1 (Goedert et al., 2001; Wakabayashi et al., 2000). LBs contain components of the ubiquitin-proteasome system, the major pathway for the degradation and clearance of

redundant proteins such as mutant, misfolded, denatured, damaged and short-lived regulatory proteins (Lennox, Lowe, Morrell, Landon and Mayer, 1998). These components include ubiquitination and deubiquitination enzymes, proteasomal subunits and proteasome activators (McNaught, Shashidharan, Perl, Jenner and Olanow, 2002; Li, Nijhawan, Budihardjo, Srinivasula, Ahmad, Alnemri and Wang, 1997; Lowe, McDermott, Landon, Mayer and Wilkinson, 1990).

LBs also contain heat shock proteins, such as HSP70 and HSP90 that have a key role in protein transport, degradation and folding (McNaught et al., 2002; Auluck, Chan, Trojanowski, Lee and Bonini, 2002).

The proteins which constitute LBs may be nitrated (Good, Hsu, Werner, Perl and Olanow, 1998), oxidised (Castellani, Perry and Siedlak, 2002), ubiquitinated or phosphorylated (Fujiwara, Hasegawa and Dohmae, 2002). The granular material in the centre of the Lewy body is considered to be comprised of ubiquitinated protein aggregates (McNaught et al., 2002) and the radiating filaments of the periphery are formed at least in part from fibrillar α-synuclein and neurofilaments (Spillantini, Crowther, Jakes, Cairns, Lantos and Goedert, 1997).

Figure 1.2 The Lewy neurite and Lewy body

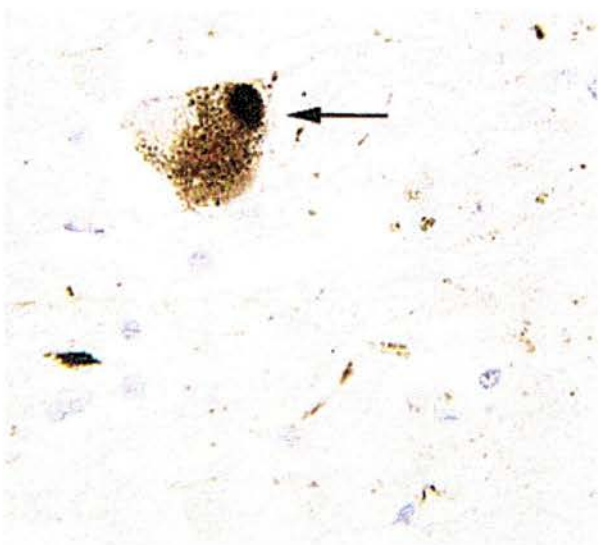
(a) Image shows a Lewy neurite from a human parkinsonian brain stained with anti-α-synuclein antibody (arrow).

(b) A Lewy body again from a human parkinsonian brain, stained with anti-α-synuclein antibody, highlighting the extent of α-synuclein throughout the structure (arrow).

Images reproduced with permission from www.saigata-nh.go.jp

a

b



1.5.2 Fibril formation and cytotoxicity

Fibrillated α-synuclein has subsequently been identified as a major component of Lewy bodies found in dopaminergic neurones of the SNpc (Spillantini, Schmidt, Lee, Trojanowski, Jakes and Goedert, 1997). Moreover, it has been demonstrated that α-synuclein can aggregate *in vitro* into filaments structurally similar to those found in pathological inclusions and mutated forms of human α-synuclein aggregate substantially faster than wild-type protein (Conway et al., 1998, 2000; Narhi, Wood, Steavenson, Jiang, Wu, Anafi, Kaufman, Martin, Sitney, Denis, Louis, Wypych, Biere and Citron, 1999; Serpell, Berriman, Jakes, Goedert and Crowther, 2000). These results suggest a causative role for α-synuclein aggregation in the development of certain neuropathologies. At the same time it was shown that α-synuclein could cause cell death or render cells more sensitive to toxic insults independently of protein fibrillation (Lee et al., 2001; Ostrerova et al., 1999; Petrucelli et al., 2002). For example, over-expression of α- but not γ-synuclein in cultured nodose neurones has shown that both wild-type and the A53T and A30P mutant forms can be neurotoxic without fibril formation (Saha, Ninkina, Hanger, Anderton, Davies and Buchman, 2000).

In certain cases, more prominent effects of mutant α-synuclein on cell survival have been demonstrated. Cultured dopaminergic neuroblastoma SH-SY5Y cells expressing either wild-type, A53T or A30P α-synuclein all displayed elevated levels of reactive oxygen species (ROS) levels, the mutated forms more so (Junn and Mouradian, 2002). In addition upon exposure of these cells to dopamine, the cells expressing mutated α-synuclein showed a much greater reduction in viability (Junn and Mouradian, 2002). Furthermore, similar studies using truncated forms of α-

synuclein revealed that proteins with a truncated C-terminal showed the greatest susceptibility to neurotoxic insult (Kanda, Bishop, Eglitis, Yang and Mouradian, 2000). The aberrant over-expression of human α-synuclein has also been shown to produce toxic effects in primary culture of human dopaminergic neurones, the A53T mutated form being particularly neurotoxic (Zhou, Hurlbert, Schaack, Prasad and Freed, 2000).

Significantly, it has been demonstrated that both β- and γ-synuclein have the ability to inhibit α-synuclein protofibril formation *in vivo* and despite having similar biophysical properties neither β- or γ-synuclein display any fibril formation activity (Uversky, Li, Bower and Fink, 2002). It has been shown that in transgenic models expressing human α-synuclein, mice develop a parkinsonian movement disorder, concurrent with nonfibrillar α-synuclein inclusions and the loss of dopaminergic nerve terminals. Mice expressing both human α- and β-synuclein show significant amelioration of all neurotoxic effects, with β-synuclein preventing the formation of toxic protofibrils (Hashimoto et al., 2001).

β-synuclein has subsequently been shown to act as a negative regulator of α-synuclein preventing its inhibition of ubiquitin-independent proteosomal degradation as discussed above (section 1.3.5) (Snyder et al., 2005).

The presence of elevated levels or a mutated form of α-synuclein is sufficient to cause the accumulation of cytoplasmic dopamine which can trigger oxidative stress. α-synuclein is appearing as the key molecule in this downward cycle, as presence of its mutated forms in familial cases or accumulation of the wild-type protein within the cell appears to be cytotoxic, possibly as a consequence of dopamine induced oxidative stress (Martin, Williamson, Paleologou, Allsop and El-Agnaf, 2004).

These findings have fostered the hypothesis that the balance of synucleins is critical *in vivo*, where β- and γ-synuclein act synergistically with the toxic α-synuclein.

1.5.3 Oxidative stress mechanism of neurotoxicity

Oxidative stress is a damaging condition resulting from insufficient scavenging of reactive oxygen species (ROS), which are restrained under normal physiological conditions. The efficient anti-oxidant systems that scavenge these damaging molecules may become impaired as part of the normal aging process or under pathological conditions which can cause irreversible damage. Nigral dopaminergic neurones are particularly sensitive to oxidative stress as the metabolism of dopamine produces a number of molecules that can act as endogenous toxins. For example, dopamine can auto-oxidise at physiological pH into toxic dopamine-quinone species, superoxide radicals and hydrogen peroxide (Graham, 1978).

Reactive oxygen species cause functional alterations to proteins, lipids and DNA. Damage to protein and DNA are obviously undesirable and damage to lipids results in loss of lipid integrity and increased permeability to ions such as calcium, which can promote excitotoxicity (Halliwell, 1998). As dopamine is a neurotransmitter, under normal circumstances it would be sequestered into vesicles and thus cells are protected from the deleterious effects of dopamine. In PD, nigral cells show a heightened state of oxidative stress, highlighted by increased levels of lipid by-products, protein and DNA oxidation and compensatory increases in antioxidant systems (Jenner, 1998; Floor and Wetzel, 1998). Conversely, glutathione, a co-substrate in the detoxification of hydrogen peroxide by glutathione peroxidase, is markedly decreased in the SN of PD patients (Alam, Jenner, Daniel, Lees, Cairns,

Marsden, Jenner and Halliwell, 1997). The predisposition of this region to oxidative stress is evident in rats where a dietary deficiency of the antioxidant vitamin E leads to a 33% reduction in the number of TH positive cells in the *substantia nigra*, leaving other brain area un-effected (Dexter, Nanayakkara, Goss-Sampson, Muller, Harding, Marsden and Jenner, 1994).

1.6 The role of synucleins in the development and function of the *Substantia Nigra* (SN)

A considerable weight of evidence implicates the synuclein family in neurodegeneration, particularly within the SN, making this population a critical target for further study. The shift in intracellular compartmentalisation of γ-synuclein seen in the *substantia nigra* during development highlights the necessity for further study.

1.6.1 The *substantia nigra*

The midbrain (mesencephalon) is the smallest and most rostral part of the brainstem, the centre of which is the tegmentum, containing nuclei involved in the modulation of movement. These nuclei include the SN and the red nucleus, in addition the oculomotor nucleus and its accessory nucleus, the Edinger-Westphal nucleus (Carpenter, 1976). The entity described as the SN is in fact composed of two distinct structures, the *pars reticulata* (SNpr) and the *pars compacta* (SNpc). The SNpr is the ventral aspect of the SN, a subnucleus that contains large amounts of the enzyme glutamate decarboxylase required for the synthesis of gamma-amino butyric acid (GABA) by its complement of neurones (Korotkova, Ponomarenko, Brown and Haas, 2004). The neurones in the SNpr project principally to the thalamus (ventral anterior, ventral lateral, and dorsomedial nuclei) but also to brain stem nuclei (superior colliculus, pedunculopontine nucleus) and use GABA as their neurotransmitter (Beckstead, Domesick and Nauta, 1976; Korotkova et al., 2004). The SNpc is the dorsal aspect of the SN, with neurones projecting primarily to the neostriatum which use dopamine (DA) as a neurotransmitter. They also contain the

black pigment neuromelanin, a product similar to dopamine that is produced from tyrosine (Obeso JA, Rodriguez-Oroz, Rodriguez, Macias, Alvarez, Guridi, Vitek and DeLong, 2000).

The ventral tegmental area (VTA) lies directly adjacent to the SN, the cells of which also use DA as its primary neurotransmitter. Axons arising from the dopaminergic neurones of the VTA form the mesolimbic pathway, which innervate the nucleus accumbens and the olfactory tubercle. Recently, it has been suggested that changes in the limbic system can contribute to schizophrenia and other psychological disorders (Meyer-Lindenberg, Miletich, Kohn, Esposito, Carson, Quarantelli, Weinberger and Berman, 2002). Together the SN and VTA comprise the largest concentration of the excitatory transmitter dopamine (DA) in the mammalian CNS (Nelson, Liang, Sinton and German, 1996). However, the SN and the VTA are not the only structures which utilise dopamine as their neurotransmitter; there are in fact eight groups of dopaminergic cells in the mammalian CNS identified by the nomenclature A8-17 (Table 1.2).

The SNpc neurones which innervate the striatum are implicated in Parkinson's disease (PD), as described above (section 1.4.3). A slow progressive loss of the dopaminergic neurones within the SNpc leads to a diminished release of dopamine in the striatum. When this reaches a critical threshold level comprising a loss of 70-80% of striatal DA, the clinical symptoms of PD appear. To reach this threshold level approximately 40% of tyrosine hydroxylase (TH, the rate limiting enzyme in dopamine production) positive neurones of the SNpc will have become dysfunctional (Bezard, Dovero, Prunier, Ravenscroft, Chalon, Guilloteau, Crossman, Bioulac, Brotchie and Gross, 2001).

The fully developed dopaminergic neurones of the substantia nigra play their physiological role as a part of the basal ganglia (BG) (Fig. 1.3). The basal ganglia include the caudate nucleus, lentiform nucleus, putamen, pallidum, sub-thalamic nucleus and the substantia nigra. A feedback network of neuronal connections exists between the constituents of the BG, with the dopaminergic, GABAergic and glutamatergic neurons exerting excitatory and inhibitory control on the system. The caudate nucleus and the putamen make up the corpus striatum, the largest structure in the basal ganglia and as a unit constituting a major centre in the extrapyramidal motor system. It is the putamen which receives the majority of inputs from the SNpc hence the depletion seen in nigrostriatal dopamine. The current understanding is that the two pathways exist to modulate the tonic stimulation of the motor areas of the cerebral cortex by the thalamus and are therefore involved in the control of normal voluntary movements (Fig 1.4) (Middleton and Strick, 2000). Disorders relating to the BG tend to manifest as an inability to initiate voluntary movement and inability to suppress involuntary movement, abnormal velocity and amount of movement as well as disturbances in muscle tone. The archetypal example of this is Parkinson's disease (Murray, Sepic, Gardner and Downs, 1978).

To gain a more complete understanding of how the synucleins may affect cell survival and or physiology in normal and neuropathological conditions it is important to consider how these structures and their constituent parts are formed.

Table 1.2 Mammalian dopaminergic cell groups

Table outlines the known dopaminergic cell groups, their reference and location.

Mammalian dopaminergic cell groups		
Group	**Location**	**Region**
A8	Retro-rubral nucleus	Mesencephalic
A9	Substantia nigra pars compacta	Mesencephalic
A10	Ventral tegmental area	Mesencephalic
A11	Caudal hypothalamus	Diencephalic
A12	Arcuate infundibular nucleus	Diencephalic
A13	Zona incerta	Diencephalic
A14	Medial rostral hypothalamus	Diencephalic
A15	Olfactory tubercle	Telencephalic
A16	Olfactory bulb	Telencephalic
A17	Retina	Telencephalic

Figure 1.3 Anatomy of the basal ganglia

A diagramatic illustration of the structures which make up the basal ganglia. Image from Bear, Connors and Paradiso (2001).

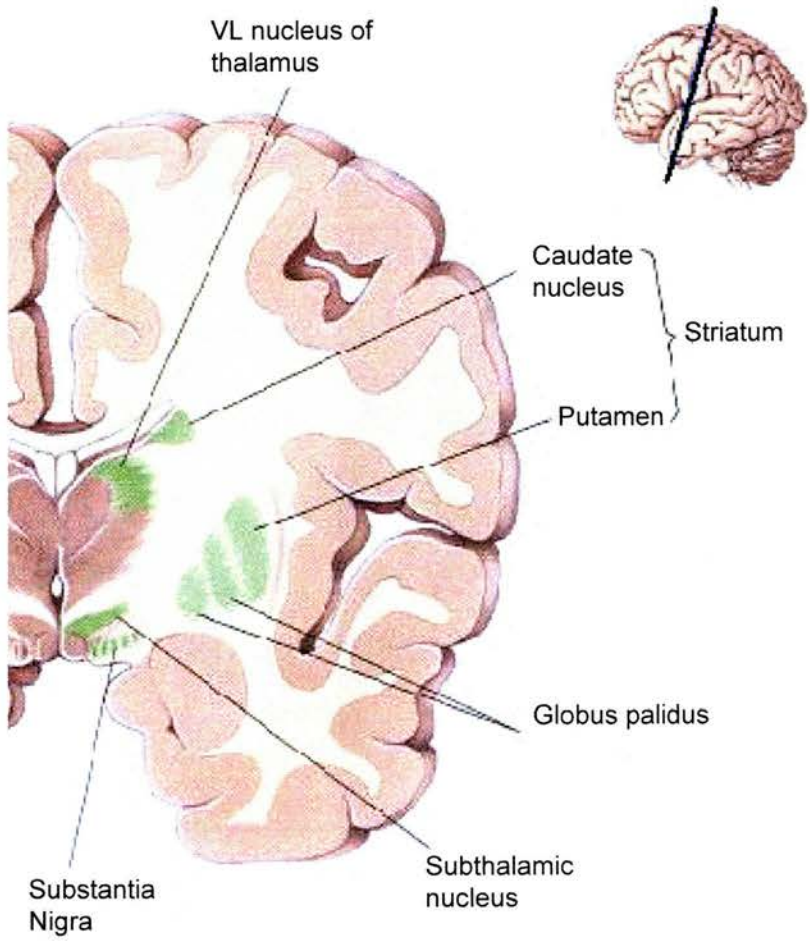
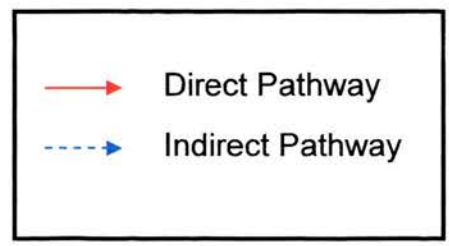
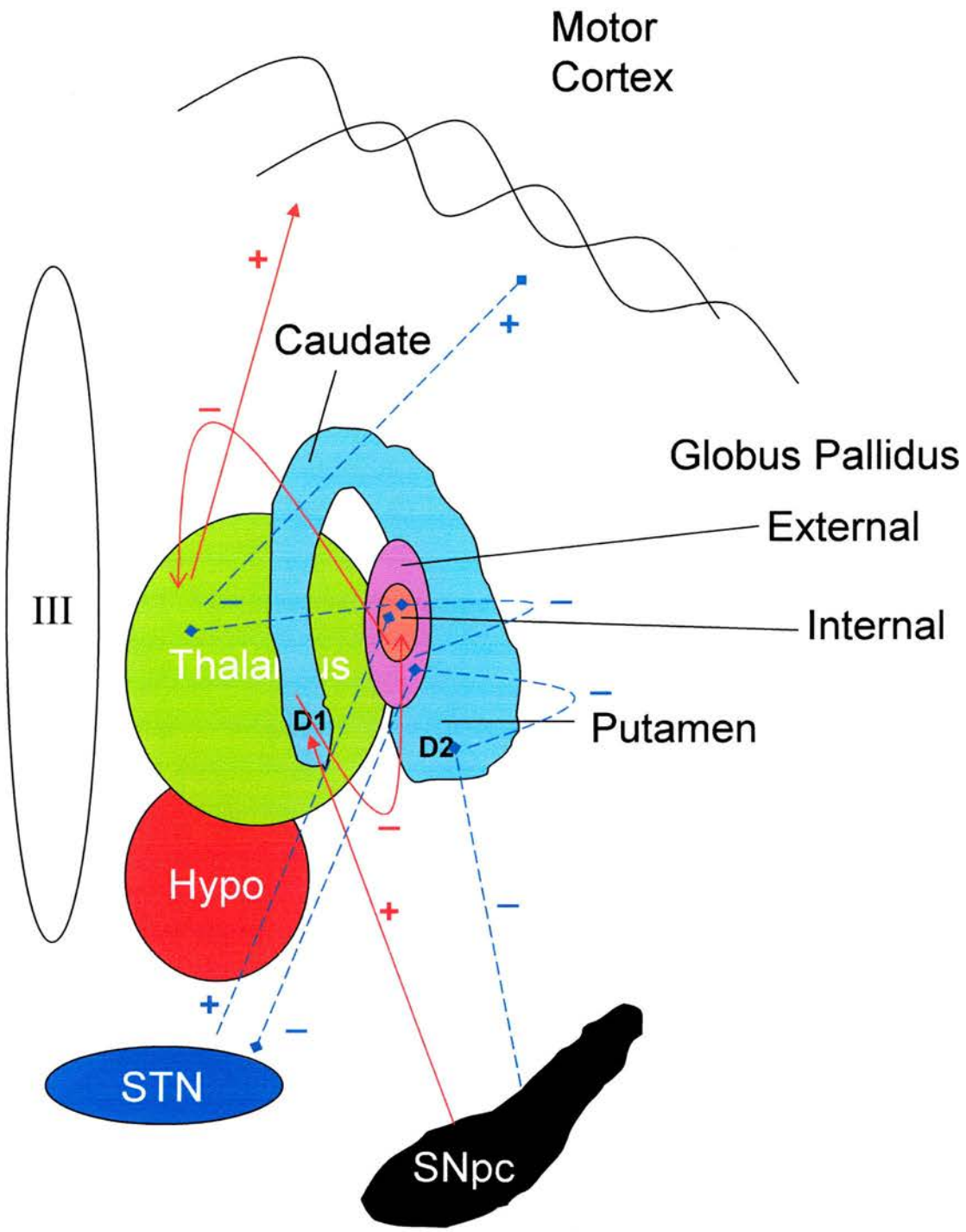

VL nucleus of thalamus
Caudate nucleus
Striatum
Putamen
Globus palidus
Subthalamic nucleus
Substantia Nigra

Figure 1.4 Pathways of the basal ganglia

The projections from the medium spiny neurons of the caudate and putamen to the internal segment of the globus pallidus and substantia nigra are part of the “direct pathway” and serve to release the upper motor neurons from tonic inhibition. A second pathway serves to increase the level of tonic inhibition and is called the "indirect pathway". This pathway provides a second route linking the corpus striatum with the internal globus pallidus and substantia nigra. In the indirect pathway, another population of medium spiny neurons projects to the lateral or external segment of the globus pallidus. This external division sends projections to both the internal segment of the globus pallidus and the subthalamic nucleus of the ventral thalamus. But, instead of projecting to structures outside of the basal ganglia, the subthalamic nucleus projects back to the internal segment of the globus pallidus and to the substantia nigra. These latter two nuclei project out of the basal ganglia, which thus allows the indirect pathway to influence the activity of the upper motor neurons.

Abbreviations: D1/2 - dopamine receptors; Hypo - Hypothalamus ; STN - Sub thalamic nucleus; SNpc - substantia nigra pars compacta; III – Third ventricle.




Motor
Cortex
Caudate
Globus Pallidus
External
Internal
Putamen
III
Thala
us
D1
D2
Hypo
STN
SNpc

1.6.2 Embryogenesis and development of the CNS and dopaminergic neurones

The CNS arises from the neuroectoderm, which is generated shortly after gastrulation in the developing embryo. After a period of thickening, the neuroectoderm begins to roll up along its long axis to form the neural tube, which, upon closure, subdivides to form the fore-, mid- and hind-brain. These processes are mediated by diffusible growth factors emitted from structures such as the floor plate, notochord, non-epidermal ectoderm and later, by roof plate cells (Lee and Jessell, 1999). It is from this primitive midbrain, more accurately the anterior neuroectoderm that the dopaminergic neurones will eventually differentiate. The majority of mouse dopaminergic neurones are thought to become post-mitotic at around embryonic day 12 (E12) and only 24 hours later TH begins to be synthesised and results in dopamine production (Foster, Schultzberg, Kokfelt, Goldstein, Hemmings, Ouimet, Walaas and Greengard, 1988).

Differentiation advances with the outgrowth of the first processes towards their target fields and the formation of axonal terminals by E18. To date, three independent pathways have been identified which play a role in the differentiation of DA neurones. The key transcription factors controlling these pathways are Nurr1, Lmx1b and the engrailed genes En1 and En2.

Nurr1 is expressed by 95% of all TH-positive, thus dopamine-producing, midbrain neurones and is thought to influence the use of dopamine as their neurotransmitter (Backman, Perlmann, Wallen, Hoffer and Morales, 1999). In the absence of Nurr1, several proteins concerned with the synthesis, axonal transport, storage, uptake and reuptake of dopamine are also absent. These key proteins include TH, aromatic

amino acid decarboxylase, vesicle membrane associated transporter and dopamine transporter (DAT). Mice lacking Nurr1 appear to develop normally but are unable to feed and die shortly after birth confirming its importance (Castillo, Baffi, Palkovits, Goldstein, Kopin, Witta, Magnuson and Nikodem, 1998). Significantly, TH and DAT have been shown to interact with α-synuclein, as discussed previously (section 1.3.2).

Lmx1b is a member of the homeodomain family of proteins and is widely expressed throughout the body, including in skeletal, ocular and renal as well as neuronal tissues. Expression begins at E7.5 in several locations including the region of the ventral midbrain that will give rise to dopaminergic neurones. If this factor is absent, very few TH-positive cells are present during development and are completely absent by E16 (Smidt, Asbreuk, Cox, Chen, Johnson and Burbach, 2000). Lmx1b is thought to have a crucial role in the production and maintenance of Wnt1, a proto-oncogene critical to patterning during midbrain development, the loss of which, results in the complete absence of the midbrain (Adams, Maida, Golden and Riddle, 2000).

The engrailed genes En1 and En2 are also homeodomain transcription factors that are expressed from E8 in patches in the anterior ectoderm and from E12 are expressed constantly within dopaminergic neurones (Simon, Saueressig, Wurst, Goulding and O'Leary, 2001). These patches fuse and form a line of cells which will later become the border between mid- and hindbrain regions. Only double mutant animals lacking both En1 and En2 show any developmental deficit with respect to midbrain dopaminergic neurones (Liu and Joyner, 2001). Cells generated in the ventral midbrain of double mutant mice begin to generate TH but fail to progress any further through development and by E14 are lost. These mice show a deletion of the

midbrain and anterior hindbrain and die shortly after birth (Joyner, Herrup, Auerbach, Davis and Rossant, 1991). Interestingly, α-synuclein expression is thought to be regulated in part by the engrailed genes, as α-synuclein expression is reduced at E12 in En1 mutants and is absent altogether in the remaining midbrain dopaminergic cells of double En1/2 mutants (Simon et al., 2001).

1.6.3 Natural cell death

Dopaminergic cell numbers continue to increase during a period of mitosis lasting from E11 to E15 in the SN and migration of these cells is complete at E18. This period of growth and differentiation is followed by a period of natural cell death (NCD). In mice, this event begins around the time of birth and is completed by postnatal day 30 (P30). The wave of apoptotic cell death peaks at P2, trailing off until P14 when a second smaller peak occurs (Jackson-Lewis, Vila, Djaldetti, Guegan, Liberatore, Liu, O'Malley, Burke and Przedborski, 2000).

The NCD of SN neurones is exclusively apoptotic and is thought to be mediated via the intrinsic pathway which initially involves the release of the electron transfer protein cytochrome c from its mitochondrial inter-membrane location into the cytoplasm (Liu, Kim, Yang, Jemmerson and Wang, 1996). Mounting evidence suggests that this release is mediated by members of the Bcl-2 family of proteins (Adams and Cory, 1998). Fifteen member proteins exist in mammals, which include pro-apoptotic proteins such as Bak, Bim, Bad, and Bax; and members with anti-apoptotic activity like Bcl-2 and Bcl-X_L. Bax, Bak, and Bid have been reported to directly cause the release of cytochrome c from mitochondria (Jurgensmeier, Xie, Deveraux, Ellerby, Bredesen an Reed, 1998; Kuwana, Smith, Muzio, Dixit,

Newmeyer and Kornbluth, 1998; Li, Zhu, Xu and Yuan, 1998). Once released into the cytoplasm, cytochrome c forms a multimeric complex with Apaf-1 (Li et al., 1997, Zou, Henzel, Liu, Lutschg and Wang, 1997), a 130 kDa protein with a caspase recruitment domain (Zou et al., 1997). This complex activates procaspase-9 which in turn activates caspases 3, 6, and 9. Caspases are proteins which carry out the proteolytic cleavage which brings about the death of a cell. The term caspase is an amalgamation of cysteinyl aspartate-specific proteinases, highlighting the enzymes' characteristic feature of cleaving their substrates after aspartic acid residues. The morphological hallmarks of apoptosis, chromatin condensation, DNA fragmentation, and breakdown of the nuclear membrane are the direct consequence of caspase activity (Nicholson and Thornberry, 1997; Thornberry and Lazebnik, 1998).

In transgenic mice, over-expressing the anti-apoptotic protein Bcl-2 under the control of the TH promoter, causes a reduction in NCD in the SN, with increased numbers of TH-positive cells in adult animals (Jackson-Lewis et al., 2000). These findings support the theory that the intrinsic pathway of NCD operates within the SN. In addition, Bax null mutant animals show a trend for diminished levels of NCD in the SN lending more support to this hypothesis (Vila, Vukosavic, Jackson-Lewis, Neystat, Jakowec and Przedborski, 2000). Activated caspase-3 and caspase cleavage products have been observed in apoptotic profiles of NCD in SN nuclei (Jeon, Kholodilov, Oo, Kim, Tomaselli, Srinivasan, Stefanis and Burke, 1999; Vila, Jackson-Lewis, Vukosavic, Djaldetti, Liberatore, Offen, Korsmeyer and Przedborski, 2001). However, caspase-3 may not be the essential mediator of NCD in the dopaminergic neurones as caspase-3 null mutant mice do not display any reduction

the level of NCD seen in SN neurones (Oppenheim, Flavell, Vinsant, Prevette, Kuan and Rakic, 2001).

The cell death discussed above occurs normally during development in response to a lack of neurotrophic support, enabling neuronal populations to regulate their size. There is a great weight of evidence suggesting that glial cell-line derived neurotrophic factor (GDNF) is responsible for regulating the survival of the nigro-striatal dopaminergic neurones. GDNF was identified as a protein capable of supporting the development of embryonic mesencephalic dopamine neurones in tissue culture (Lin, Doherty, Lile, Bektesh and Collins, 1993). GDNF mRNA is present in the striatum and is expressed at its highest levels during early postnatal development, with protein expression being highest post partum (Schaar, Sieber, Dreyfus and Black, 1993; Lopez-Martin, Caruncho, Rodriguez-Pallares, Guerra and Labandeira-Garcia, 1999). GDNF can also be transported retrogradely from the striatum to the SNpc and the GDNF receptor, GFRα1 as well as the tyrosine kinase Ret are both expressed in the SNpc (Tomac, Widenfalk, Lin, Kohno, Ebendal, Hoffer and Olson, 1995; Glazner, Mu and Springer, 1998).

However, mice lacking GDNF itself and GFRα1 null mutant mice show no deficit in the number of dopaminergic neurones in the SN at birth (Enomoto, Araki, Jackman, Heuckeroth, Snider, Johnson and Milbrandt, 1998; Rosenthal, 1999). These animals die very early on during the period of natural cell death, likely due to other adverse effects such as enteric nervous and renal system abnormalities, hence any mesencephalic effects may not be identifiable (Pichel, Shen, Sheng, Granholm, Drago, Grinberg, Lee, Huang, Saarma, Hoffer, Sariola and Westphal, 1996). More recently, it has been shown that only GDNF was capable of supporting postnatal

dopaminergic neurones *in vitro* by suppressing apoptosis (Burke, Antonelli and Sulzer, 1998).

1.7 Synucleins and cancer

The synuclein family have been implicated in pathologies outside the nervous system, were the focus of attention shifts from α- to γ-synuclein. High throughput cDNA screening revealed that γ-synuclein (BCSG-1) (Table 1.1) was expressed at a dramatically increased level in breast cancer tissue (Ji et al., 1997). In normal tissue, γ-synuclein expression is at a very low level and this is also the case in benign breast carcinomas. Expression increases slightly in ductal carcinomas but a dramatic rise is seen in highly malignant, infiltrating tumours (Bruening et al., 2000). This increase in expression led to the hypothesis that γ-synuclein may be linked to tumour progression from benign to the highly malignant stage (Ji et al., 1997). Further investigations using western blotting revealed that 82% of all ductal carcinomas studied expressed either β- or γ-synuclein or both, but no expression was detected in normal tissue, and no detection of α-synuclein was reported under either condition (Bruening et al., 2000). Immunohistochemical analysis of ovarian epithelial cells has shown no expression of any synuclein in normal ovarian tissue (Bruening et al., 2000). Expression of at least one member of the synuclein family was detected in 87% of ovarian carcinoma samples studied with 42% showing the presence of all three (Bruening et al., 2000). Interestingly however, γ-synuclein expression was also observed in 20% of preneoplastic lesions of the ovary, including epithelial inclusion cysts, hyperplastic epithelium and papillary structures (Bruening et al., 2000).

There is considerable evidence to suggest that γ- synuclein has an important role in tumour progression in both breast and ovarian tumours. Evidence supporting this has recently been reported, whereby γ-synuclein strongly stimulated the ligand-dependent transcriptional activity of oestrogen receptor-α (ER-α) in breast cancer cells (Jiang, Liu, Goldberg and Shi, 2004). The stimulatory effect of γ-synuclein on ER-α-regulated gene expression and cell growth could be effectively inhibited by anti-estrogens (Jiang et al., 2004). These data indicate that γ-synuclein is required for efficient ER-α signalling and thus, stimulated hormone-responsive mammary tumours.

1.8 The generation of γ-synuclein null mutant mice

The previous body of synuclein research has been disproportionably swayed toward α-synuclein. However, β- and γ-synuclein have also been implicated in pathological conditions. Given the homology displayed between these proteins, any information relating to their physiological functions could have critical importance to the development of any therapeutic strategies for the diseases with which they are associated.

To this end, a line of γ-synuclein null mutant mice ($\gamma^{-/-}$) were produced in our laboratory by Dr N. Ninkina and Dr V. Buchman (Ninkina, Papachroni, Robertson, Schmidt, Delaney, O'Neill, Court, Rosenthal, Fleetwood-Walker, Davies and Buchman, 2003) to gain a greater understanding of any effect of γ-synuclein on murine physiology and development. These animals were used during the course of this project and their generation is described below. Subsequently these animals were

used to generate α-/γ-synuclein double null mutant mice but this will be discussed later (Chapter 3, section 3.5.7).

1.8.1 Generating chimeras

To achieve the null mutation, first three coding exons from the γ-synuclein locus were replaced with a Neomycin resistance cassette in mouse ES cells (Fig. 1.5). This was achieved via homologous recombination between the targeting vector and ES cells from 129Ola mice. During the selection of clones with correct homologous recombination, southern blotting was performed (Fig. 1.6) using DNA taken from clones transformed with the targeting cassette. Those found to carry a mutant allele were selected for further use. The chosen clones were then injected into blastocysts harvested from C57Bl6 mice before being transferred into pseudo-pregnant females to develop.

Several chimeric mice were generated from three independent ES cell clones. These chimeras were interbred with C57Bl6 mice to prove their ability to transfer the ES cells genotype to the next generation and produce heterozygous animals, from which the first true γ-synuclein null mutants were generated, by interbreeding the heterozygous progeny. The resulting animals were then screened to ensure that possession of two copies of the mutated allele precipitated a loss of functional γ-synuclein protein (Chapter 3, section 3.3).

Figure 1.5 Deletion of exons I, II, III and promoter region of the mouse γ-synuclein gene by homologous recombination

The organisation of the wild-type genomic locus (top), targeting vector (middle) and the resulting knock-out locus (bottom) are shown. The restriction endonuclease sites are designated as: E, EcoR1; B, BamH1; Xb, XbaI; K, Kpn1; Bg, BglIII. Hybridisation probes a, b and c, which were used for the analysis of homologous recombination are also shown.

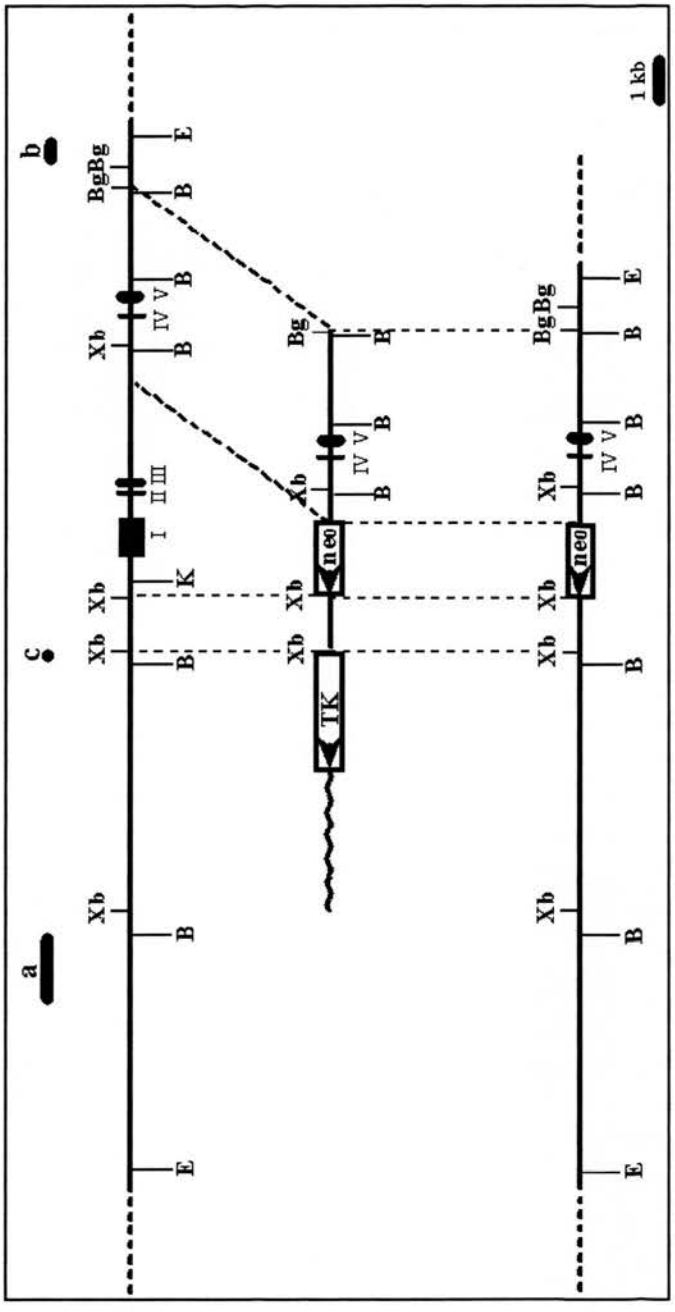
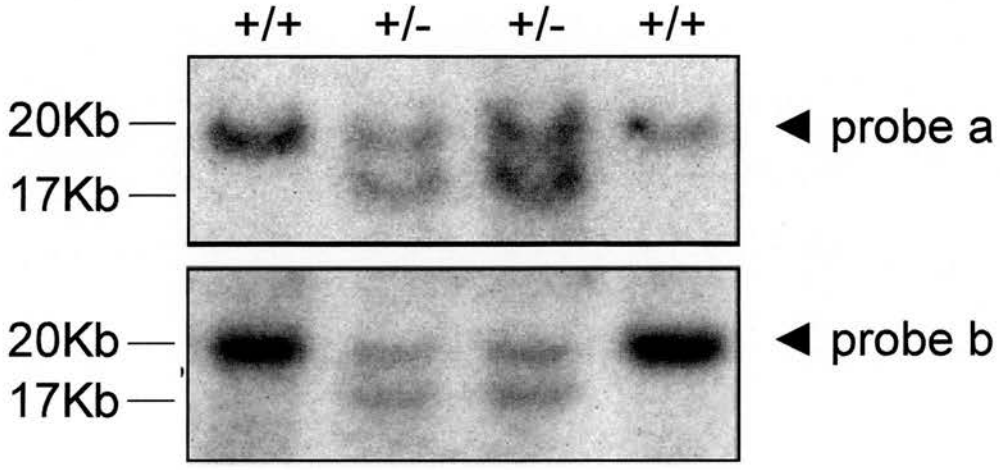

a
b
c
1 kb
BgBg
E
B
IV V
Xb
Bg
R
II III
I
K
neo
TK

Figure 1.6 Southern hybridisation showing an example of the analysis of ES cell clones

DNA from four neomycin-resistant embryonic stem (ES) cell lines were digested with EcoR1 and hybridised with either probe a or b. Only a 20 kb wild-type (+/+) band was detected in two clones with random insertion of PGK-neo cassette and the homologous recombination in two other clones resulted in the detection of a 17 kb band.


+/+
+/-
+/-
+/+
20Kb
17Kb
probe a
20Kb
17Kb
probe b

1.9 Hypotheses & Aims

1.9.1 Hypotheses

- A targeted disruption of the γ-synuclein gene in *mus musculus* which results in loss of protein may precipitate phenotypic abnormalities as the gene is expressed during development.
- Phenotypic abnormalities arising from the γ-synuclein mutation may be exacerbated in the absence of both α- and γ-synuclein.
- The balance of the synuclein proteins is thought to have importance in neuronal survival. Thus any alteration of this balance may cause increased sensitivity of neurones to neurotoxic insult.

1.9.2 Aims

- To produce a highly specific mouse-γ-synuclein antibody in order to investigate protein localisation and verify mRNA expression data.
- To investigate the effects of synuclein null mutations on selected neuronal populations shown to express γ-synuclein.
- To test the sensitivity of synuclein null mutant neurones to various forms of neurotoxic insult.

Chapter 2: Materials & Methods

2.1 Reagent supplier information

Company	Product
Amersham Biotech, Ltd., Little Chalfont, Buckinghamshire, HP7 9NA	ECL kit, Hybond N^+, Hybond-P nylon membrane, Kodak β-max hyperfilm
AstraZeneca Ltd., Macclesfield, Cheshire	Fluothane
BDH Chemical Company, Merck House, Poole, BH1 1TD	DPX, Vectamount, Polysine Slides, Paraffin Wax, Triton X100
Charles River Ltd., 251 Ballardvale Street, Wilmington, MA 01887-1000	C57Bl6 wild-type females
Chemicon International Ltd., 2 Admiral House, Cardinal Way, Harrow, HA3 5UT	Sheep anti-α-synuclein antibody
Erie Scientific Company, Portsmouth, NH03801, USA	Gold seal ultrastick slides
Fisher Scientific UK Ltd., Leicestershire, LE11 5RE.	3MM filter paper, glacial acetic acid
Gibco Life Technologies Ltd., Inchinnan Park, Paisley, PA4 9RP	All tissue culture reagents
MBI Fermentas, Hanover, MD 21076, USA	DNA ladder
Novocastra Laboratories Ltd, Balliol Business Park West, Benton Lane Newcastle upon Tyne, NE12 8EW	Tyrosine Hydroxylase Antibody
Pharmacia (Pfizer), Sandwich, Kent, CT13 9NJ	G50 Sephadex column
Pierce Biotechnology Inc., Rockford, IL 61105 USA	BCA protein assay reagent kit
Promega UK Ltd., Delta House, Chilworth Reaearch Centre,	PCR Primers, PCR buffer, $MgCl_2$, Taq Polymerase

Southampton, SO16 7NS	
Qiagen Ltd, Qiagen House, Fleming Way Crawley,West Sussex, RH10 9NQ	Machery Nagel DNA purification kit
Raymond A Lamb, 6 Sunbeam Road, London NW10 6JL	0.5% cresyl violet stain
Sigma-Aldrich Company Ltd., Fancy Road, Poole, Dorset, BH12 4QH	All other reagents
Supelco, Sigma-Aldrich Company Ltd. Dorset, BH12 4QH	Hi-Trap affinity column
Thermo Hybaid (Interactiva Div.) Thermo Hybaid GmbH, Sedanstraβe 10 D-89077, Ulm, Germany	PCR Primers
Thermo Electron Corporation **Shandon Products Ltd.,** Chadwick Rd, Astmoor Industrial Estate, Astmoor, Runcorn, Cheshire WA7 1PR	Sequenza staining system
Ugo Basile, Biological research apparatus, Via G. Borghi 43 21025 Comerio VA - Italy	5200 Rotarod I.R. activity monitor
Vector Laboratories, Ltd., 3, Accent Park, Bakewell Road, Orton Southgate, Peterborough, PE2 6XS	ABC antibody detection kit, biotinylated anti-sheep IgG, Vectamount, biotinylated horse anti-mouse IgG

2.2 Buffers and solutions

Denhardt's Solution 100X
10g Ficoll 400
10g Polyvinylpyrrolidone
10g Bovine Serum Albumin
ddH_2O to 500ml

Formamide/Formaldehyde solution
1ml formamide
330µl formaldehyde

Laemmli Lysis Buffer
0.652M Tris-Cl, pH 6.8; 2% (w/v) SDS
0.1% (w/v) bromophenol blue
50% (v/v) glycerol

PBS (Phosphate Buffered Saline) 10X
80g NaCl
2g KCl
14.4g Na_2HPO_4
2.4g KH_2PO_4
800ml ddH_2O
pH to 7.4, ddH_2O to 1L, autoclave

SSC (pH 7.0) 20X
175.3g NaCl
88.2g Sodium citrate
ddH_2O to 1L

Striping solution for membranes
0.1X SSC
0.1%SDS

RNA Gel Loading Buffer
25% Glycerol
300µg/ml ethidium bromide
MOPS

Guanidine buffer
5M Guanidine thiocyanate
100mM Tris pH 7.5
10mM EDTA pH 8.0
7µl/ml β-mercaptoethanol

MOPS buffer
0.05 M Sodium Acetate
0.01 M EDTA
0.2 M MOPS, pH 7.0

RNA Hybridisation Buffer
50ml De-ionised formamide
5ml ssDNA (5mg/ml)
1ml EDTA (0.5M)
3ml NaP (1M pH7)
5ml SDS (10%)
25ml 20X SSC
ddH_20 to 100ml

STES buffer
10 mM Tris-HCl, pH 7.5
100 mM NaCl
1 mM EDTA
2% sarkosyl

TAE buffer
48.4g Tris-base
10.9g Glacial Acetic Acid
2.92g EDTA
1L ddH_2O

Western blot Running buffer 10X

Tris	30.25g
Glycine	144g
SDS (10%)	10ml
ddH_2O to 1L	

Western blot Transfer buffer

2.5mM Tris
15mM Glycine
20% Methanol

2.3 Generation and maintenance of experimental synuclein null mutant mice

2.3.1 γ-synuclein null mutant mice

A male chimera was successfully generated as described above (Chapter 1, section 1.8). This animal was crossed with wild-type female C57Bl6/J mice to check for germline transfer of ES cells genotype. The progeny had a mottled coat colour indicating that germline transfer had occurred. Following genotyping, heterozygous animals were considered as the F1 generation. Before true null mutant animals could be produced for experimental purposes, the genetic background of the animals was purified by a series of consecutive backcrosses. The male heterozygous animals from each backcross were mated with C57Bl6 wild-type females obtained from Charles River (Chapter 3, section 3.5.3). This backcrossing was repeated for six generations before heterozygous male and female animals were intercrossed to produce a heterogeneous litter containing null mutant, heterozygotes and wild-type animals (Chapter 3, section 3.5.3; Fig. 3.14). One male heterozygote animal was placed in a cage with two wild-type females, which were checked for the presence of a mucopolysaccharide plug daily to indicate mating had occurred. Once plugged, females were separated for the duration of the pregnancy and nursing.

2.3.2 α-synuclein null mutant mice

α-synuclein null mutant mice ($\alpha^{-/-}$) were gifted to our laboratory and have been described previously (Abeliovich et al., 2000). These mice were backcrossed with C57Bl6/J mice for at least six generations before setting up intercrosses.

2.3.3 α-/γ-synuclein double null mutant mice

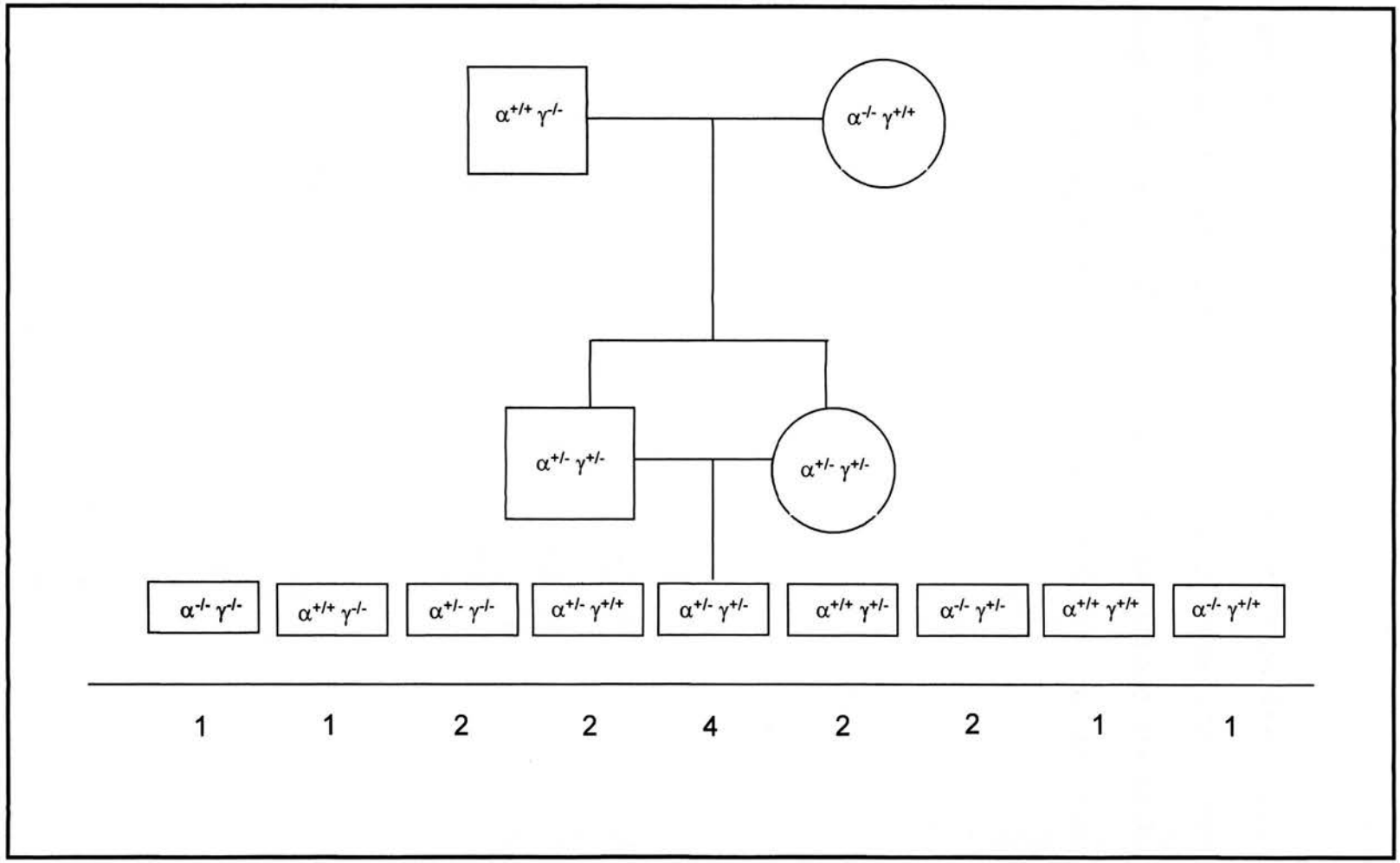
In order to generate double null mutant animals from those described above, a planned program of genotyping and breeding was undertaken. F6 α- and γ-synuclein null mutants were intercrossed, thus producing a litter of animals which were heterozygous for both genes ($\alpha^{+/-}/\gamma^{+/-}$). Interbreeding these male and female littermates created the possibility of a one in sixteen chance of being homozygous for both the null mutant alleles ($\alpha^{-/-}/\gamma^{-/-}$) (Fig. 2.1).

2.3.4 Animal care and regulations

All procedures relating to animals were carried out under and according to the UK Animals (Scientific Procedures) Act 1986. Animals were housed in accordance with Home Office regulations, given access to food and water *ad libitum* and had a 12 hour light/dark cycle.

Figure 2.1 Diagram illustrating the creation of α-/γ-synuclein double null mutant mice

Diagram illustrates the one in sixteen chance of creating a double mutant from heterozygous parents and shows all the other possible genotypes. The litters produced on average followed the classic Mendelian distribution for two non-linked alleles which is indicated numerically below


$\alpha^{+/+}\gamma^{-/-}$
$\alpha^{-/-}\gamma^{+/+}$
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$\alpha^{+/+}\gamma^{-/-}$
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$\alpha^{+/+}\gamma^{+/+}$
$\alpha^{-/-}\gamma^{+/+}$
1
1
2
2
4
2
2
1
1

2.3.5 Genotyping transgenic animals

Genotyping was an essential part of maintaining each colony, as every effort was made to minimise the number of mice utilised and also to determine the genotypic status of the animals used. In order to collect a DNA sample from each mouse, a small tail biopsy was taken from each animal under a general anaesthetic (Flurothane). At the same time each animal was given a numbered ear tag to accurately identify the animal and match it to the correct genotype. Tail biopsies were transferred to labelled eppendorf tubes, numbered corresponding to the ear tags and refrigerated until needed.

To extract and purify DNA from these samples, a kit-based protocol was utilised following the manufacturer's (Qiagen Ltd.) instructions. Initially, 200µl of a Proteinase K in lysis buffer solution was added to each tissue sample and digested at 56°C overnight. This tissue lysate was vortexed and put through the column-based purification system as per the instructions.

2.3.6 Polymerase chain reaction (PCR)

In order to amplify the correct alleles in each case, a combination of primers was chosen and mixed in the same reaction to assay either the presence or absence of the wild-type or mutated allele of α- or γ-synuclein. Primers were chosen within the wild-type and mutated loci as well as a primer that was common to both wild-type and mutated alleles for each gene.

The following primers were used to assay the wild-type and mutated γ-synuclein alleles:

SAUP:	5'-AGT CCT GGC ACC TCT AAG CA-3'
GDN:	5'-GGG CTG ATG TGT GGC TAT CT-3'
Neo B:	5'-GAA GAA CGA GAT CAG CAG CC-3'

The primer named SAUP is the common primer to both alleles; NeoB binds the mutated allele and GDN only the wild-type allele.

The following primers were used to assay the wild-type and mutated α-synuclein alleles:

Neo A:	5'-GAA GAA CGA GAT CAG CAG CC-3'
AKoC:	5'-CAG CGA AAG GAA AGC CGA GTG ATG TAC T-3'
aS-ups:	5'-CAG CTC AAG TTC AGC CAC GA-3'

The primer named aS-ups is the common primer to both alleles; NeoA binds the mutated allele and AKoC only the wild-type allele.

The primers were diluted to a working concentration of 15pM/µl and aliquoted so a fresh vial could be used for each session. The following were combined to create a master mix solution according to the number of samples to be genotyped. If n=1 and was to be genotyped for γ-synuclein then the following would be combined;

- PCR buffer 5µl
- GDN 1µl
- SAUP 1µl
- Neo B 1µl
- dNTP's (25mM each) 0.5µl
- ddH_20 31µl
- $MgCl_2$ 10µl
- Taq polymerase 0.7µl

50µl of the mix was added to 2µl of purified sample DNA, pre-aliquoted into a thin walled 0.5ml PCR tube and inserted into a thermal cycler and taken through the following program for 45 cycles before being transferred to 4°C:

- 95°C 45 seconds
- 56°C 30 seconds
- 72°C 60 seconds

The optimal conditions for these reactions were elucidated previously by Dr. N. Ninkina.

2.3.7 Agarose gel separation of PCR products

To visualise the results, agarose gels were used to separate the amplified DNA fragments. 1.2g of agarose was melted in 100ml of TAE buffer containing ethidium bromide (0.5μg/ml). This was mixed and heated in a microwave until boiling, then allowed to cool. When the solution had cooled sufficiently it was poured into a gel mould, a plastic comb inserted and the gel allowed to set. After approximately 30 minutes the combs were removed and the gel transferred into a gel tank filled with the TAE buffer so the buffer just covered the surface of the gel. 10μl of each PCR sample was mixed with 2μl of 6X loading buffer to aid sample loading into individual wells in the gel. A 120mA current was the passed through the gel to separate any DNA fragments by size. 2μl of a DNA ladder loaded at each side of the gel. This ladder contained DNA fragments of known size which allow the accurate estimation of fragment size when imaged. Once the dye front of the gel had neared the end of the gel, it was imaged under UV light. PCR analysis of animals resulting from intercrosses in the γ-synuclein null mutant colony yielded two distinct bands. The mutated allele produced a 397bp fragment amplified between the NeoB and SAUP primers in comparison with the WT allele with a band at 490bp. In the case of α-synuclein, the WT band measured 520bp and the mutated allele 450bp. These reactions were carried out separately for double null mutant animals. A typical result of γ-synuclein mutant colony genotyping is shown in Chapter 3 (Fig. 3.10).

2.4 Northern hybridisation

2.4.1 Preparation and isolation of RNA for northern blotting

Tissue was taken via dissection and immediately frozen in liquid nitrogen. The tissue was then ground into a fine powder using a pestle and mortar and kept cold with liquid nitrogen. 500µl of guanidine buffer was added gradually as the tissue was ground further, ensuring the lysed tissue and buffer became well mixed. Only then were lysates allowed to thaw and transferred to an eppendorf tube.

Following this, 50µl 2M sodium acetate (pH 4.0) was added and the samples vortexed thoroughly, before adding 500µl of acidic phenol and again vortexing the solution. The samples were placed on ice and 100µl of chloroform was added to each sample and vortexed intensively and then spun in a bench top centrifuge at 13,000 rpm for 10 minutes. The aqueous phase of each sample was carefully removed to a fresh tube and the RNA precipitated for 24 hours at -20°C by adding 500µl of isopropanol. After this precipitation period, the samples were spun for 20 minutes at 13,000 rpm, the supernatant removed and the resulting pellet washed in 70% ethanol. These pellets were allowed to dry in a fume hood until the ethanol had evaporated, before being re-suspended in 400µl of guanidine buffer. The process of extraction was repeated to enhance purification. Following the second phenol/chloroform extraction, samples were spun at 13,000 rpm for 10 minutes and 2.5 times the volume of ethanol were added to precipitate RNA from the supernatant over a 24 hour period at -20°C. The pellets were collected by spinning samples for 20 minutes at 15,000 rpm and washed with 70% ethanol before finally being dried under vacuum in a speed vac for approximately 25 minutes at 45°C.

In order to load equivalent amounts of RNA into each lane on the gel, the relative RNA content was estimated in each case. Initially the size of each sample pellet was estimated relative to the smallest, designated as 1 unit. Then 7µl MOPS, 14µl formamide/ formaldehyde solution for every 1 unit of pellet size was added to each sample. The RNA samples were then incubated between at 60°C for 10-15 minutes before being placed on ice and 2µl of the loading dye was added. The samples of RNA extracted from the retina were loaded onto an agarose gel. After 30 minutes running at 1000mA the gel was observed under UV light and relative amounts of RNA in samples were estimated from the intensities of rRNA bands. These estimates were used to normalise amounts of RNA in samples before loading a preparative gel for Northern blot analysis.

2.4.2 Preparation of a formaldehyde agarose gel

For the gel, 2g of agarose was added to 88ml of distilled water, boiled in a microwave and allowed to cool to 55°C. 32ml of 5X MOPS buffer and 40ml of 38% formaldehyde were pre-warmed to 55°C and combined with the cooling gel. This was poured into a gel tray with combs and allowed to set. The combs were removed and formaldehyde/1X MOPS buffer mixture 1:3 was used to fill the wells. The tank was subsequently filled with 1X MOPS buffer to the top of the gel. Samples were loaded and run on the gel at 100mA until the dye front had travelled to the bottom the gel and an image was captured. Before blotting the gel was washed in 20X SSC for approximately 40 minutes, changing the solution twice.

2.4.3 Filter preparation

A piece of Hybond N^+ membrane was cut to the size of the gel and rinsed in boiling ddH_2O. The membrane was cooled and washed in 2X SSC before being placed in contact with the gel ensuring there were no air bubbles present. This was placed in a tank containing 2X SSC. A 2.5cm pile of absorbent paper was placed on top of the membrane to draw fluid through the gel carrying the RNA onto the filter and a weight was placed onto of this stack to keep it stable. After approximately 12 hours, the filter was removed, washed in 2X SSC, allowed to air dry and was finally baked in an 80°C oven under vacuum. The RNA was cross-linked to the filter via exposure to 354nm UV light. The filter was then ready for hybridisation.

2.4.4 Probe DNA labelling with ^{32}P

In order to label a cDNA probe, a nick translation reaction was performed. During this reaction a DNAse made nicks in the dsDNA causing damage which is subsequently repaired by the DNA polymerase, thus incorporating ^{32}P labelled cytosine into the particular DNA sequence. As radioactive materials were being used full safety precautions were observed at all times in accordance with university regulations.

The nick translation reaction mixture contained the following:

- $\alpha[^{32}P]$ dCTP (10mCi/ml) 10µl
- cDNA (0.1mg/ml) 0.1µl
- ddH_20 19µl
- 5X(-C)dNTP buffer mix 8µl
- DNaseI 10ng/ml 1µl
- DNApolI 10u/µl 1µl

The above reaction was mixed and allowed to run for 30 minutes at 16°C. The efficacy of incorporation was then tested by placing a sub µl aliquot onto a small piece of GF/B filter paper. The level of activity was measured using a Geiger counter then the paper was washed with trichloroacetic acid (TCA). The activity level was re-measured and if it had not diminished significantly (i.e. no more than 60% after washing), the reaction was stopped by adding STES buffer to make up the reaction volume to 150µl. However, the probe purification was necessary before it could be used in a hybridisation. A standard G50 Sephadex nick-column was used for the desalting and gel filtration of the probe. The cap was removed from the column and the buffer drained and discarded, before being washed through twice with 3ml washes of STES buffer and again the flow-through discarded. 150µl of the reaction mixture was loaded onto the column. The column was washed with 400ml of STES and the resulting flow-through checked for absence of radioactivity before being discarded. Another 400µl of STES buffer was added onto the column and the flow-through collected into an eppendorf tube. The presence of the probe in this fraction was assessed using a positive radioactivity signal and reserved. 10µl tRNA (10mg/ml) was added to the purified probe fraction to act as a carrier for the effective precipitation of the DNA. In addition, 1/20 probe volume of 3M sodium acetate (pH 5.5) and 1ml 100% ethanol were added. The contents of the tube were mixed and kept at -20°C for 12 hours to allow the DNA to precipitate out fully. Following this period, it was spun at 13,000 rpm for 5 minutes, the ethanol removed and the pellet dried then re-suspended in 100µl of sterile dH_2O. The probe was then denatured by adding 5µl volume 10M sodium hydroxide and incubating for 10 minutes at 68°C.

This separated double stranded DNA into single stranded DNA. The probe was neutralised with 50µl of 1M acidic Tris before it was added to a hybridisation buffer.

2.4.5 Hybridisation

The filter membrane was placed in a secure tube inside the hybridisation oven and allowed to pre-hybridise in buffer for 2-3 hours at 42°C. The buffer was refreshed and the probe added and allowed to hybridise with the filter for 48 hours at 42°C. Following this hybridisation, the waste buffer was disposed of in accordance with the university regulations for the disposal of radioactive waste.

The filter was then rinsed in 2X SSC at room temperature and washed in 2X SSC containing 0.2% SDS at 68°C for 1 hour, changing the buffer twice. The filter was then wrapped in cling film and transferred to a cassette for exposure with X-ray film. The membrane was placed in contact with the film and both were sandwiched together between two phospho-luminescent screens inside the cassette, which was then transferred into -70°C. After 12-16 hours, the film was developed and if necessary, fresh film replaced in the cassette for a longer exposure. In order to re-hybridise the membrane with a different probe, the membrane was boiled in a striping solution (0.1X SSC/ 0.1% SDS). This allowed for probes to α- and β-synuclein and the house keeping gene glyceraldehydes-3-phosphate dehydrogenase GAPDH to be re-hybridised on the same blot.

2.5 Western blotting

2.5.1 Sample preparation

The same process was used in each case irrespective of the tissue however the volume of buffer was adjusted to maintain an approximately equal protein concentration independent of the size of collected tissue samples. Tissues were taken, frozen and ground down in liquid nitrogen and homogenised in Laemmli lysis buffer, using a syringe in an eppendorf tube. Once homogenised, the samples were spun at 10,000 rpm for 10 minutes and supernatants boiled for 5 minutes to denature proteins.

2.5.2 Gel Preparation

Running buffer solution was prepared prior to the gel pouring, and the equipment cleaned thoroughly and assembled as per manufacturer's (BioRad) instructions.

	Stacking Gels	Separating Gel (12.5%)
Acrylamide	1ml	4.13ml
Tris pH 8.8	----	3ml
Tris pH 6.8	0.6ml	----
SDS (10%)	150µl	100µl
ddH_2O	4.3ml	2.5ml
Amonium persulphate 10%	150µl	100µl
TEMED	10µl	10µl

The separating gel was poured and allowed to set prior to the pouring of the stacking gel on top, and again left to set. The gel plates were then placed into the running tank and filled with running buffer. 12µl of sample solutions were then loaded into individual wells using duck-billed tips. The apparatus was then run at 200V for approximately 45 minutes or until the dye front reached the bottom of the gel.

Four pieces of 3MM paper were cut per gel and one piece of Hybond-P nylon membrane pre soaked in methanol. The paper, membrane and sponges in the blotting apparatus were equilibrated in transfer buffer. The gels were removed carefully from between the glass plates and placed in contact with the nylon membrane. The blotting apparatus was assembled in accordance with manufacturers' instructions (Biorad) and run at 26v for 90 minutes. After the completion of the transfer process the nylon membrane was removed and intensively washed in PBS before being blocked in 10% skimmed milk powder in PBST (PBS + 0.1% Tween) for 1 hour at room temperature on a rocker.

The primary antibody dilution was made in a 3% milk/PBST solution, applied to the membrane and incubated for 12-18 hours at 4°C again on a rocker. The membrane was then washed several times with fresh PBST for 1 hour prior to the application of an appropriate HRP-conjugated secondary antibody diluted in the same 3% milk/PBST solution, for 1 hour at room temperature shaking gently. After washing thoroughly with PBST, the blot was incubated with 2ml of enhanced chemiluminescence (ECL) solution (1ml A + 1ml B; ECL kit). The solution was discarded and the membrane wrapped in cling film and placed in a cassette in contact with Kodak β-max hyperfilm for between 1-20 minutes. The film was then developed allowing protein bands to be visualised.

2.6 Histology

2.6.1 Sample collection

Animals were prepared following standard Schedule 1 protocols; CO_2 in rising concentration until dead or by cervical dislocation. To remove the brain, the skull

was exposed and an incision was carefully made along the midline beginning at the rostral cervical vertebrae and extending along the sagittal suture, perpendicular to which transverse cuts were made. In all animals, brains were excised after breaking the parietal and temporal bones and cutting the cranial nerves, placed into a tissue cassette and directly into an appropriate fixative for immersion fixation.
Embryonic tissue we immersed whole or decapitated and only the head placed inside the immersion cassette. Spinal cord tissue taken for dorsal root ganglion (DRG) analysis was dissected out from post-natal animals, leaving rib roots as reference makers for the identification of L6 DRG. No perfusion was employed at all during the course of this work.

2.6.2 Optimisation

Prior to each different immunohistochemical investigation, an optimisation trial was carried out, as the same antibody can give widely differing results depending on which fixation technique is employed. For instance, Carnoy's solution is alcohol based and can cause substantial damage to cell membranes, hence antibodies raised against cell surface antigens may prove ineffective. Conversely, formaldehyde-based fixatives such as paraformaldehyde (PFA) or neutral buffered formalin (NBF) can cross-link proteins, masking antigens and therefore require an antigen unmasking step to be included in the processing discussed below. Therefore, four widely used fixatives were tested with each new antibody:

1. Carnoy's solution (60% ethanol, 30% chloroform, 10% glacial acetic acid)
2. 10% neutral buffered formalin (NBF) as stock
3. FAA 18% NBF, 62% alcohol, 4% glacial acetic acid, 16% ddH_20)

4. 4% Paraformaldehyde (PFA) in PBS

2.6.3 Sample processing

Before the tissues were embedded in paraffin wax, all traces of fixative were removed. In the case of formalin-based fixatives, this was achieved by using two hour long changes of distilled water before overnight dehydration at room temperature in 75% ethanol. This step was not required for alcohol-based fixation as the further dehydration in a graded alcohol series was employed. This involved three 10 minute long changes of 96% ethanol followed by two 30 minute changes of 100% ethanol. As the paraffin wax, which was the ultimate destination for these tissues, is not miscible with alcohol, this was replaced with chloroform. The samples were taken through one change of 1:1 alcohol/chloroform before being immersed in chloroform for one hour then, changing the solution, left for 24 hours. Following this period, the samples in their respective cassettes, were taken through three 1 hour long changes of molten, filtered paraffin wax maintained at 58°C. Following removal from the cassettes, brain samples were transferred into a mould and the wax was allowed to solidify around them. This was performed at 4oC as it lends a finer crystalline structure to the wax compared to room temperature cooling.

The solid sample blocks were then trimmed and cut to a thickness of 8μm on a microtome (Microm 310), with the wax strips floated out onto a 55oC water bath and collected onto microscope slides (Ultrastick, Gold Seal). The cut sections were then allowed to dry for a period of no less than 24 hours at 37°C to ensure maximum adherence.

Sections were floated out onto slides in one of two fashions. If more than one staining technique needed to be applied to one particular structure, the *substantia nigra* for example, strips were alternated between two slides, creating two sets (Fig. 2.2a). This allowed us to directly compare consecutive sections stained in different ways. Conversely, if the entire sample was to be stained uniformly then consecutive strips were mounted on single slides (Fig. 2.2b).

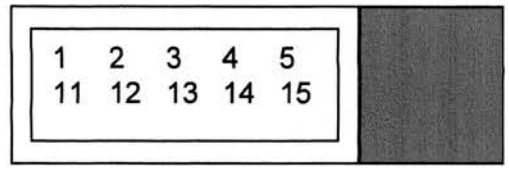
A common protocol was followed during all paraffin-based histology to remove all traces of wax and prepare the sections for incubation with a specific primary antibody or the application of other histological stains. Initially, slides were placed in stainless steel racks and taken through solutions in 350ml glass troughs. The initial step was two 5 minute changes of fresh Xylene, to remove all traces of wax from the sections. It was essential to remove all traces of this organic solvent so that water-based solutions could be applied subsequently. This was accomplished by taking the slides through a graded alcohol series, first two changes of 100% absolute alcohol of 5 minutes each, then a further 3 minutes in both 96% and 70%, respectively. The sections were then introduced into distilled water for 5 minutes, removing all traces of alcohol prior to a further 5 minute wash in PBS.

Figure 2.2 Differential positioning of sections for histology

(a) Illustration of section layout for immunohistochemistry when two sets of slides were required; one for marker and another for target protein. Adjacent sections can be compared.

(b) Section layout for histology using one staining technique or antibody. Slides are mounted sequentially producing one set of slides for the same treatment.

a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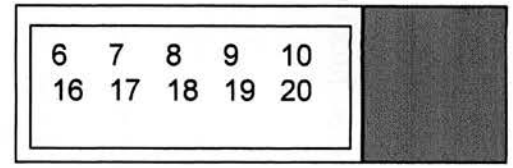
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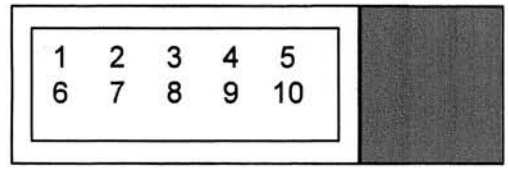
b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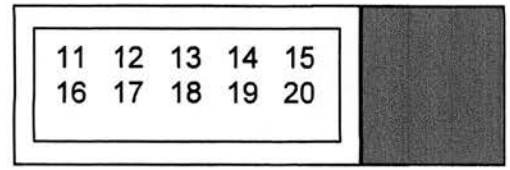

11 12 13 14 15
16 17 18 19 20

2.6.4 Immunostaining

The next stage in the processing depended on the fixative used and future staining, as antigen retrieval may be required. As mentioned, formalin-based fixation has the effect of cross-linking proteins masking antigens hence interfering with the staining process. The chosen method for antigen retrieval was a microwave based technique in a 0.1M citric acid solution (pH 6.0). Slides were transferred to a plastic rack and incubated for approximately 10 minutes before being placed in the microwave. The rack in solution was heated to 90°C over a period of 10 minutes and boiling of the solution was avoided.

If a DAB reaction was to be carried out for the visualisation of a primary-secondary complex via the avidin-biotin system, a quenching solution was required to quench the endogenous peroxidase activity in the tissue. This quench consisted of a 70% methanol solution in PBS, 3% hydrogen peroxide, and slides were immersed in the solution for 20 minutes at room temperature. After PBS washing, the slides were transferred into the Sequenza staining system. Slides were then incubated in 100µl of blocking solution (10% serum in PBS containing 0.4% triton X100) for no less than 60 minutes at room temperature.

The species from which the serum was chosen was dependent on the species in which the secondary antibody was raised in order to minimise non-specific interactions. A primary antibody(ies) were diluted in the same solution and applied to the slides. Incubations were carried out at 4°C for 12-16 hours.

Primary Antibody	Working Dilution
Anti-Tyrosine Hydroxylase	1:200
SK23 rabbit anti-γ-synuclein	1:50

If a fluorescent secondary antibody was to be used then the sections were washed in PBS thoroughly then the fluorophore applied for approximately 60 minutes and the slides once again washed before being mounted in Vectamount and sealed with clear nail varnish.

If a DAB protocol was being followed the procedure was more complicated. After incubation with a biotinylated secondary antibody for 60 minutes, the sections were washed with PBS and the Vector Labs ABC reagent was applied, having been prepared 30 minutes prior to use. The ABC system relies on the affinity of the glycoprotein avidin for the vitamin biotin for its action. The biotinylated secondary antibody forms a complex with the primary, bound to the antigen, to which the avidin in the ABC solution can bind. This was rinsed off with PBS after 30 minutes and the DAB applied for a maximum of 5 minutes until the coloured precipitate was produced, the peroxidase catalysing the transformation of DAB into an insoluble compound. The slides were then immersed in distilled water and taken backwards through the alcohol series and again through two changes of xylene prior to being mounted in DPX and left for 24 hours to dry.

2.7 Production of an affinity purified antibody and Immunohistochemistry

2.7.1 Production and purification of GST-fusion recombinant proteins from *E. coli*

A plasmid in which the full length coding region of mouse γ-synuclein was cloned in frame with glutathione S-transferase (GST) was produced in the laboratory earlier. BL21 bacteria were transformed with this plasmid as this particular strain has very low protease activity, allowing good protein yield. Protein production was induced by the addition of ITPG into a 500ml culture, grown over 12-16 hours. Protein was harvested after the lysis and sonication of the bacteria, allowing the protein to be recovered from the centrifuged lysate. The recovered GST-γ-synuclein was then purified using a G-sepharose bead column.

2.7.2 Affinity column preparation

To purify the antibody from the antiserum, a Hi-Trap affinity column (Supelco, Sigma-Aldrich Co.) with immobilised antigen was prepared.

The column was first washed 3 times with 2ml of ice cold 1mM HCl. Immediately following this, 1mg of purified recombinant protein was loaded onto the column and allowed to equilibrate for 30 minutes at room temperature. Before the antiserum could be loaded it had to be purified to remove any large particles and desalt it. This was achieved using a PD10 gel filtration column. The antiserum was washed through the column with 25ml of PBST and collected, and was now ready for loading onto the prepared affinity purification column.

2.7.3 Antibody purification

The purification began with the column being washed with 3ml of PBST before being equilibrated 3ml of elution buffer (glycine, pH 2.6), followed by a second wash with 10ml of PBST. The antiserum was pumped through the column twice at a rate of 0.5ml/minute collecting the flow through. The column was then rewashed with 10ml of PBST before 3ml of elution buffer was used to elute the bound antibody fraction. The column was washed again, ready for the next cycle of purification which was repeated a total of 4 times, yielding 4 antibody fractions. On collection, the antibody fractions were carefully transferred into dialysis bags and allowed to dialyse initially in PBS for 48 hours at 4°C and then in a 50% glycerol/ PBS solution at 4°C for 36 hours, changing the solution at 24 hour intervals. The addition of glycerol increased the stability of the antibody, which was then frozen in 100µl aliquots at -80°C until ready for use.

2.7.4 Tyrosine Hydroxylase (TH) staining of dopaminergic neurones

Before experimental samples were stained, an optimisation trail was carried out to determine the optimal dilution of commercial anti-TH primary antibody for immunohistochemistry on paraffin sections of mouse brains fixed with Carnoy's solution. A 1:50 dilution was found to be optimal. A 1:200 dilution of a biotinylated horse anti-mouse antibody was used as a secondary in conjunction with ABC amplification/detection system as described above (section, 2.6.4). A boundary between SNpc and VTA when assaying neuronal number was drawn after consulting anatomical atlases (Fig. 2.3). Cells were counted using the fractionator stereology method, described below (section 2.8.2).

Figure 2.3 Drawing boundaries between the SNpc and VTA in TH stained sections

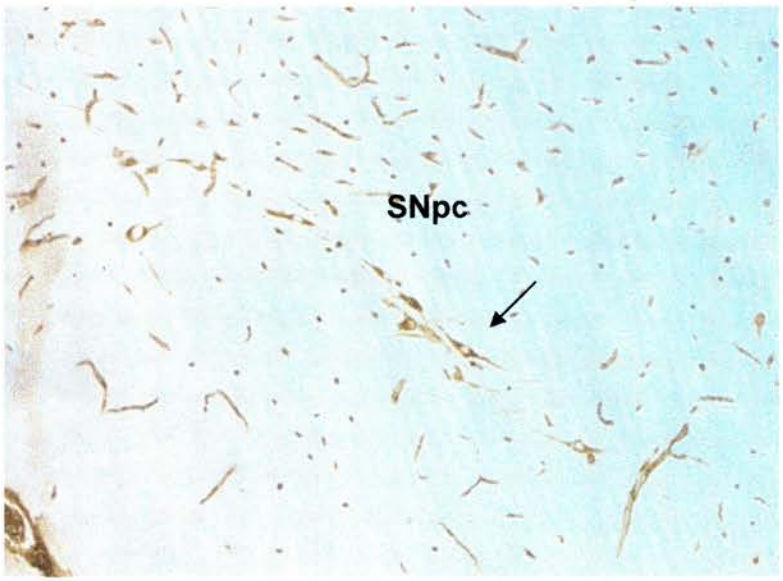
(a) Shows the first TH positive dopaminergic cells of the rostral aspect of the SNpc in parsagittal section. Counting began with these cells.

(b) As you progress caudally through the SNpc, the cell number increases and the characteristic elongated wing-like shape (outlined in red) of the structure appears (black arrow) in parasagittal section. The fibres leaving the cell bodies run out parallel to the long axis of the structure. This is highlighted (box). The line represent where the boundary between the SNpc and the VTA was drawn. VTA cells (red arrow) have fibres running off perpendicular to those of the SNpc. The line was drawn through the sparse fibrous region, largely devoid of cell bodies, separating the two structures. It is this region coupled with fibre direction which decides the boundary.

(c) Caudally, the medial lemniscus (ML) passes through the SNpc, this is not to be confused with the fibrous area separating the SNpc and VTA. Once again SNpc cells (black arrow) are separated from those of the VTA (red arrow) by both the fibrous gap and fibre direction.

(d) The cells of the SNpc become sparser until finally only the VTA is left. The boundary becomes more defined as you move caudally.

a



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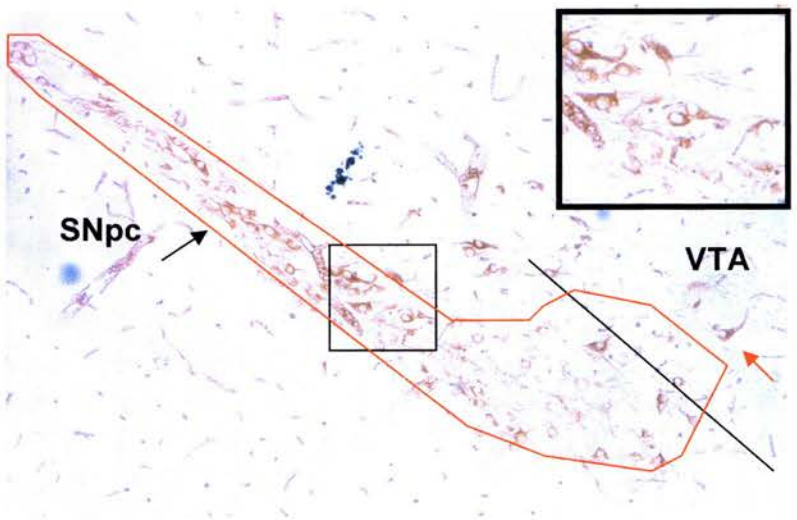
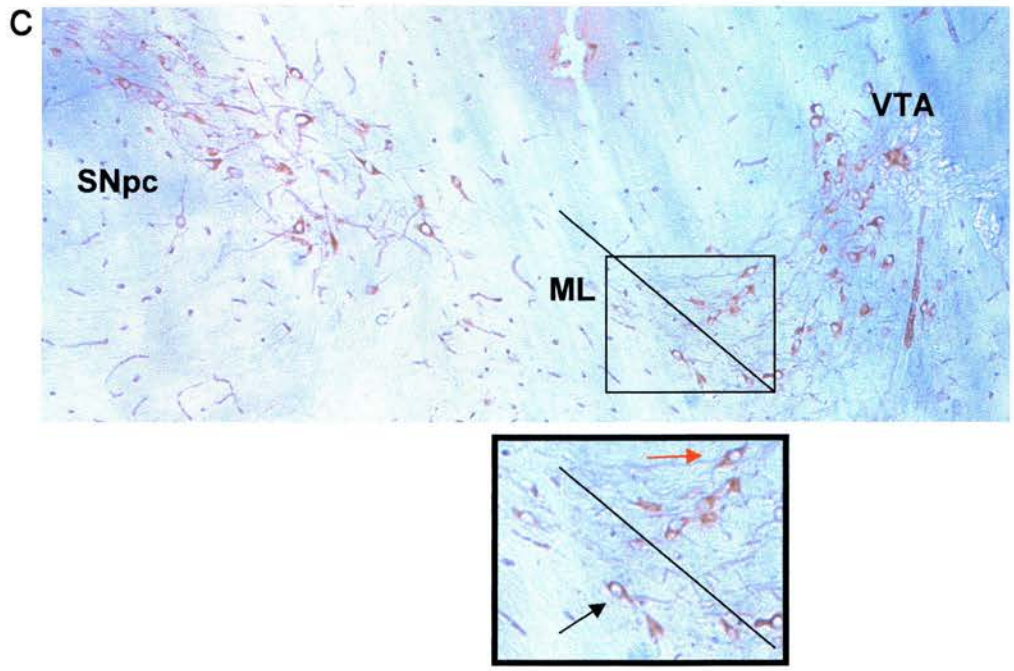
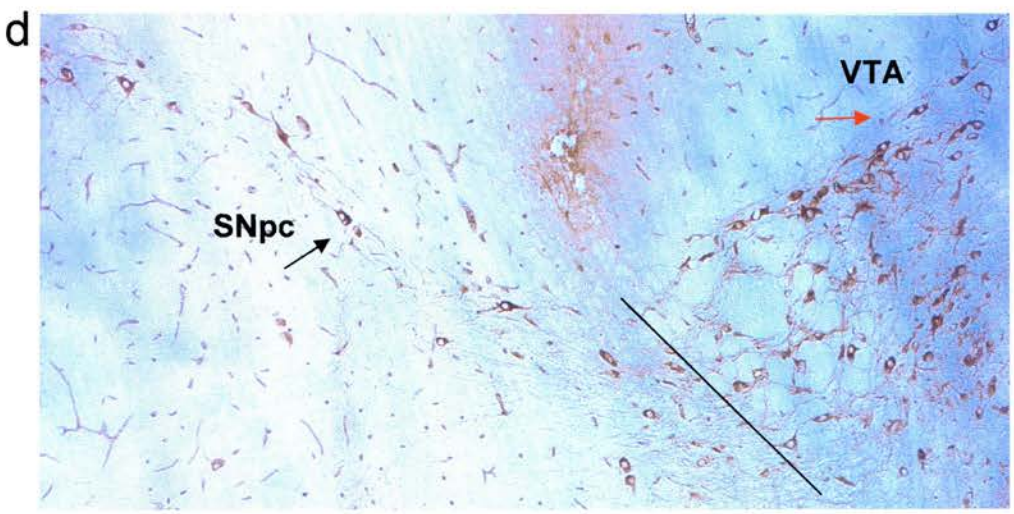



c
SNpc
VTA
ML


d
SNpc
VTA

2.8 Morphometric studies

2.8.1 Identification of sensory neurones using Cresyl Fast Violet

The L6 dorsal root ganglion (DRG) and the trigeminal ganglion (TG) were the sensory structures chosen to investigate protein localisation and assay any changes in neuronal number in sensory structures at E12, E15 and P2 in the mouse. Embryos were dissected from the uterus following schedule 1 procedures and placed directly into tissue processing cassettes. For samples taken for TG investigations, heads were removed and processed separately. The spinal columns from P2 mice were fixed and embedded as described above (section 2.6.3). Serial 8μm thick longitudinal sections were cut and mounted onto poly-lysine coated slides. The specimens were cut into sagittal as well as coronal sections. In the case of whole embryo sections, it was not possible to obtain true coronal sections throughout the entire CNS due to the curved position of the embryo.

Slides were taken down to water as described above (section 2.6.3) and two drops of glacial acetic acid were added to the 250ml of water in the staining jar. The 0.5% cresyl violet stain was made up following the manufacturers' instructions (Raymond A Lamb) 24 hours in advance of use. 250ml of stain were filtered and acidified with 3 drops of glacial acetic acid and the slides immersed for 20-25 minutes until staining was at the required intensity. The staining was then rapidly dipped into 70% then 96% ethanol until differentiated as required. This was followed by two 30 second immersions in 100% ethanol and two 5 minute changes of xylene and the slides were mounted in DPX mounting medium.

Lumbar L6 DRGs were identified by virtue of the Nissl substance and their large, round, pale-stained nuclei (Konigsmark, 1970). Neurones in the L6 DRG displaying a prominent nucleolus were counted on every eighth section. Neuronal number was quantified using a digital stereology system that employed a combination of the optical dissector and volume fraction Cavalieri methods (Kinetics Imaging, Bromborough, UK). All counts were carried out blind to animal genotypes.

To identify the TG, anatomical atlases were utilised (Theiler, 1989; Kaufman, 1992; Altman and Bayer, 1995; Paxinos and Franklin, 2001; personal communication Prof A. Davies). Cells were counted in an identical manner to the DRG.

2.8.2 Cell Counts

All of the cell counts made herein were conducted using the fractionator stereological method. Stereology is the histometrical technique by which quantitative information on the volume, surface area and/or particle numbers of objects within a two or three-dimensional structure is obtained from one- or two-dimensional data (Bancroft and Stevens, 1996). The fractionator method was used as only particle numbers or cell numbers were required in this case. Described by Gundersen in 1986, it provides a direct estimate of particle number, independent of the volume of the structure the particles form or are part of (Gundersen, 1986). In this context, the number of dopaminergic neurones in the *substantia nigra* could be estimated accurately without calculating the volume of the structure in advance.

The greatest advantage of this technique is that bias is not introduced due to shrinkage of the tissue. This shrinkage is an inevitable consequence of the fixation, processing, de-paraffination and staining procedures, despite all care being taken.

The removal of paraffin wax alone is said to cause a collapse of up to 60% of the tissue sections height (Reed and Howard, 1998). Such changes are impossible to account for under normal circumstances, hence a method that is as independent of such errors is highly desirable. Every effort was taken to collect experimental tissues together, in an attempt to ensure all aspects of fixation, processing and staining were as near chemically and chronologically identical as possible.

When DAB is used as a substrate for the peroxidase detection reaction the TH antibody stained the cell body with a dark brown precipitate leaving a clear nucleolus, thus we were able to count the large clear space surrounded by this dark stain. These cells were counted on every tenth section. Every cell in the section that lay within the determined boundaries of the SNpc and the VTA was counted.

As the cell body is thicker than the plane of section the same cell will be present in more than 1 section. To minimise the error which this introduces into cell counts Abercrombie's correction was applied.

Abercrombie's correction states that,

$$\mathbf{P = A \times M / (L + M)}$$

P being the true number of cells, A the crude number counted, M the section thickness (8μm) and L, the mean nuclear diameter.

In order to calculate L, the diameter of 100 nuclei per animal per structure were measured with the aid of a microscope, CCD camera system and the appropriate software (Axiovision, Carl Zeiss Ltd.). A mean was calculated for each animal and the correction applied. In order to make L as accurate as possible, the nuclei were chosen randomly and the distance measured was the horizontal length as they appeared on screen.

2.9 MPTP treatment

6-8 week old male C57Bl6/J mice, were weighed and treated daily with 30mg/kg doses of MPTP for 5 days, six animals from each genotypic group were present in each group. The MPTP was diluted in PBS to a concentration of 1 mg/ml and introduced via intraperitoneal (i.p.) injections. Control animals were injected with equivalent volumes of sterile PBS.

Due to the highly toxic nature of MPTP (Chapter 4, section 4.4.1), animals were housed separately from the general population of the facility. Full protective clothing was worn and any waste disposed of only after treatment with a bleach solution for three days. As urine of the subject animals must also be considered as toxic for 10 days post final injection, the bedding was also treated in this manner. 14 days after the final injection, brains were taken for histological evaluation of neurone number in both the SNpc and VTA, using the tyrosine hydroxylase immunohistochemistry method outlined above (section 2.7.4). Safety procedures were taken in accordance with published data/guidelines (Przedborski, Jackson-Lewis, Naini, Jakowec, Petzinger, Miller and Akram, 2001).

2.10 Evaluation of basic behavioural functions

2.10.1 Assessment of motor behaviour

Experimental mice were first given three training periods on a Ugo Basile (2100) accelerating rotorod.

In training sessions, a steady speed of 12 revolutions per minute (rpm) was maintained and each mouse introduced to the rotating rod and allowed to run for a

maximum of three minutes. The mice were then subsequently tested in 2 different trials. The first trial was at a constant speed of 24 rpm and the time recorded when the mouse either fell from the rod or was deemed to loose control of its movement on the apparatus. Each mouse was given 4 runs and the best three times were averaged. The lowest result was discarded in an attempt to eliminate any accidental slips which may have introduced error into these data, as excreta expelled during the test can cause slippage. Finally the mice were placed on the unit revolving at 4 rpm, and left on the rod for a maximum of 5 minutes as it accelerated to 40 rpm smoothly over that period. The same criteria as in the previous test for measuring the animals' performance on the rod were applied.

2.10.2 Assessment of general activity

General levels of activity were measured using movement in defined space over a 2 hour period. Experimental mice were introduced into a behavioural assessment chamber (Ugo Basile) for 2 hours. The activity was measured as the number of infra-red beam breaks the mouse made every 10 minutes during this period. A number of these beams cross the chamber in the x and z axis. After the time has elapsed the mouse is returned to its cage and the data taken from the machine. This test allows for the evaluation of the general activity of these mice in a foreign environment.

2.11 HPLC analysis of dopamine and its metabolites in the striatum

This work was carried out by J. Sharky and P. Jones of the Fujisawa institute of neuroscience.

Brains of 9 adult wild-type or mutant male mice on C57Bl6 background were dissected, the striatum removed on ice, snap-frozen and kept at 70°C until assayed. 95μL of 0.4 M HClO4 and 5μL of 40 lg/mL N-ω-5-HT (internal standard) was added to each thawed sample prior to sonication for 3 seconds by an ultrasonics homogenizer with a 3mm tip. The samples were centrifuged at 20,000 g on a bench top centrifuge for 25 minutes at 4°C. The pellet was frozen for protein quantification, which was carried out using a standard BCA protein assay reagent kit (Pierce, Rockford, IL, USA). Then, 50μL of the supernatant were injected onto the high performance liquid chromatography (HPLC) column through a Rheodyne injection valve connected to a 20μL loop. A BAS PM-80 solvent delivery system and BAS LC-4 ECD was used to detect dopamine (DA) and its metabolites (DOPAC, HVA and 5-HIAA). Isocratic mobile phase (75 mM sodium dihydrogen phosphate, 1.7mM octanosulphonic acid sodium salt, 100μL/L triethylamine in 90% ddH_20 water, 10% acetonitrile, pH 3.0) was used to carry the samples through a reverse phase ESA column (120 A C18 150 · 3.2 mm column packed with 3-lm particles). Flow rate was set at 0.6mL/min and the detector was at 0.7V.

2.12 Primary neurone culture

Trigeminal ganglia from P2 C57Bl6 mice were dissected in L15 media and incubated with 0.05% trypsin in $Ca^{2+/}Mg^{2+}$-free HBSS for 25 minutes at 37°C. After trypsinisation, most of the enzyme solution was removed and the ganglia washed in 2 x 10mls Hams F12 medium containing 10% heat-inactivated horse serum to remove and inactivate any residual trypsin. After washing, the ganglia were then dissociated into a single-cell suspension by trituration using a fire polished siliconised glass

pipette. Neurones were plated at low density in neurobasal medium with B27 complement, on 35 mm culture dishes containing a 12mm by12mm square, coated with poly-L-ornithine and laminin (Davies, Lee and Jaenisich, 1993).

In most cases the initial count of neurones attached within this square was carried out 3 hours after plating.

In experiments with proteasome inhibitors and metal ions, the initial count was carried out 24 hours after plating. In all cases drugs were added to cultures immediately after the initial count. The number of surviving neurones in the same area was counted 24 and 48 hours later and was expressed as a percentage of the initial count.

Cultures were prepared and treated with drugs added to the culture dishes in the following concentrations:

Proteasome inhibitors

5 μM MG-132

10 μM proteasome inhibitor I [PSI])

Heavy metal ions

30 μM CuSO4

75 μM ZnSO4

DNA-damaging agents

10 μM cytosine arabinoside [AraC]

10 μM Etoposide)

JNK signaling pathway inhibitor

20 μM SP600125

ERK signaling pathway inhibitor

20 μM PD98059

Phosphatidylinositol 3-kinase signaling pathway inhibitor

20 μM LY294002

Chapter 3: RESULTS (I)

3.1 Distribution of γ-synuclein within the murine nervous system

Previous work has demonstrated the high level of γ-synuclein mRNA expression in peripheral sensory neurones in addition to spinal and cranial motoneurones in both rat and mouse (Buchman et al., 1998). Experiments were undertaken to describe the gross overall expression pattern of γ-synuclein protein in the nervous system and investigate the intracellular localisation of γ-synuclein within certain distinct neuronal populations.

3.2 Affinity purification of mouse γ-synuclein specific (SK23) antibody

An antibody specific for mouse γ-synuclein had been previously produced and described (Buchman et al., 1998). However large quantities of the highly specific, affinity purified antibody were required to complete expression studies. Thus the antibody was purified from rabbit immune serum by affinity chromatography on a column with matrix immobilised recombinant mouse γ-synuclein.

3.2.1 Production and purification of GST-fusion mouse γ-synuclein

The initial step was to produce and purify the GST-fusion mouse γ-synuclein (Chapter 2, section 2.7). BL21 *E. coli* were transformed with a recombinant expression plasmid carrying mouse γ-synuclein cDNA cloned in-frame with glutathione-S-transferase. This plasmid was prepared previously by Dr V. Buchman.

In order to purify the fusion protein, bacteria carrying the expression plasmid were cultured in 1L of the culture medium and expression of the fusion protein was induced by adding the induction agent IPTG into the culture up to a final concentration of 0.5mM. The GST-γ-synuclein was then purified on Glutathione-Sepharose as described previously (Chapter 2, section 2.7.1). This process yielded approximately 10mg of pure fusion protein.

3.2.2 Preparation of the affinity purification column

2mg of the GST-γ-synuclein was dialysed against coupling buffer and applied to the prepared Hi-Trap column (Chapter 2, section 2.7.2). The protein concentration in the flow through fraction revealed that 50% of the loaded GST-γ-synuclein did not bind to the column matrix. This was assessed by measuring the optical density of the column flow-through. Therefore the resulting affinity purification column must have carried 1mg of the recombinant GST-γ-synuclein protein attached to the matrix.

3.2.3 Affinity purification of anti-mouse-γ-synuclein antibody from SK23 antiserum

To avoid any blockage of the affinity column with precipitous material often present in serum samples, 10ml of antiserum were first run through a PD10 gel filtration column as described above (Chapter 2, section 2.7.3). The free volume column fractions were loaded onto the GST-γ-synuclein Hi-Trap affinity column. The flow-through fraction was retained for further repeat rounds of purification. The bound antibody was eluted (Chapter 2, section 2.7.3) and the column re-equilibrated. Three further rounds of purification took place using the flow-through from the column,

until elution with the low pH buffer yielded only traces of protein, again as judged by measuring the optical density of the elutant.

Each of the four eluted fractions was then dialysed against 50% glycerol in PBS overnight to increase the stability of the protein when freezing for storage. Each fraction was checked for efficacy using both recombinant and endogenous γ-synuclein present in homogenised spinal cord lysate on western blots (Chapter 2, section 2.5) Fractions 1-4 were used individually as primary antibodies to specifically detect γ-synuclein protein on separate western blots. Four acrylamide gels were poured and samples loaded. After running the gel and transfer of proteins to the PDVF membrane, a 1:200 dilution of each fraction was applied for the required period before the washing, application of secondary anti-rabbit antibody and ECL as described above (Chapter 2, section 2.5.2). It was clear on developing the blot film that fraction 1 gave the best signal (Fig. 3.1). As expected, the efficacy dropped off the more times the flow-through was passed through the column. This can also be seen as the γ-synuclein signal appears to be fainter in fraction 2 then 3 then 4, although the same amount of protein was loaded on each blot (Fig. 3.1).

3.2.4 Optimisation of immunohistochemical detection of γ-synuclein using affinity purified antibody

As described in Chapter 2, four fixatives were tested to elucidate which was most suitable for the detection of endogenous γ-synuclein on histological sections of mouse tissue using the affinity purified anti mouse-γ-synuclein antibody and which antibody dilution was optimal for each of the fractions. Four mouse brains were dissected and fixed with one of either Carnoy's solution, Paraformaldehyde (PFA),

FAA or neutral buffered formalin (NBF) (10%) (Chapter 2, section 2.6.2). Brains were sectioned as described (Chapter 2, section 2.6.3) and 20 midbrain sections from each brain were taken for immunostaining. In addition to a negative control where no primary antibody was applied, four dilutions (1:50, 1:100, 1:250 and 1:500) of each antibody fraction were incubated on the slide before all were processed through to completion in the same way as described (Chapter 2, section 2.6.4). The best results were achieved using tissue fixed in Carnoy's with primary antibody from purification fraction 1 at a dilution of 1:50 (Fig. 3.2).

Figure 3.2(a) illustrates the specific immunostaining of endogenous γ-synuclein in the midbrian of adult wild-type C57Bl6 mice, using the antibody described above. Expression was clearly visible in the region of the SN, where intense neuropil immunostaining was evident. A negative control section taken from the same region of a $\gamma^{-/-}$ mouse confirmed the complete absence of the staining (Fig. 3.2b). No further negative control images will be shown and the absence of γ-synuclein from our null mutant animals will be discussed below (section 3.5).

Figure 3.1 SK23 affinity purified γ-synuclein antibody fractions for use in western blotting

Each of the 4 affinity purified γ-synuclein antibody fractions (1-4) were applied to western blots as primary antibodies at 1:200 dilution (a-d) to assess antibody specificity and relative concentration. Each blot contained 3 lanes, recombinant γ-synuclein (Rγ), homogenate of a wild-type mouse spinal cord containing endogenous γ-synuclein (Eγ) and homogenate of spinal cord from γ-synuclein null mutant mice ($\gamma^{-/-}$) as a negative control. Each filter was re-probed with anti-actin antibody to demonstrate that similar amounts of total proteins from wild-type and null mutant mice are present on each filter. Fraction 1 was found to have the highest antibody protein concentration with each subsequent fraction displaying less.

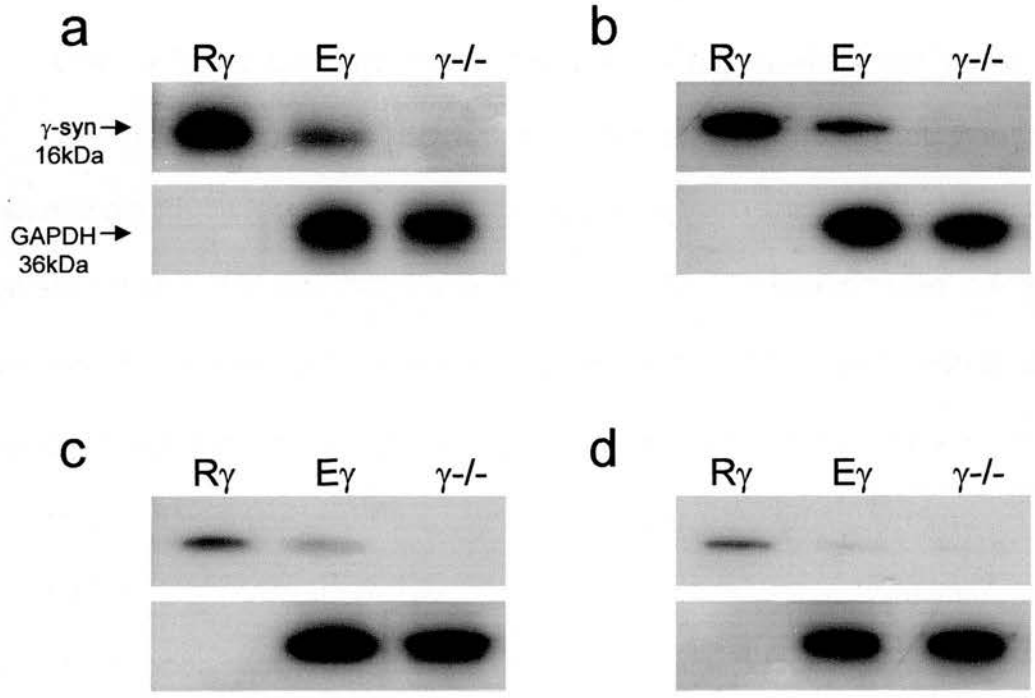
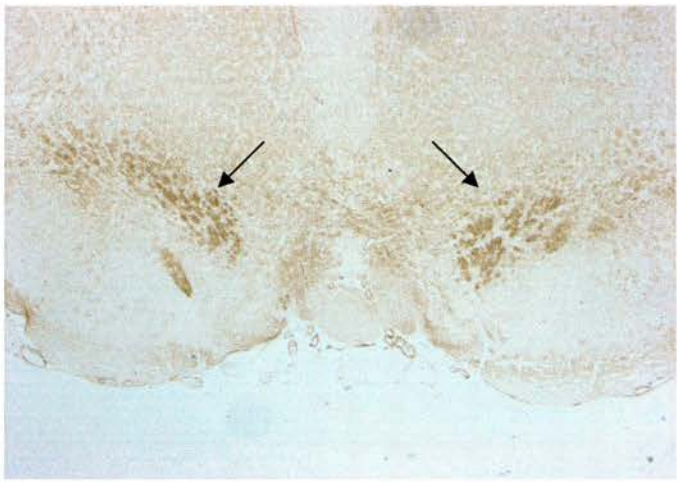

a
Rγ
Eγ
γ-/-
γ-syn→
16kDa
GAPDH→
36kDa
b
Rγ
Eγ
γ-/-
c
Rγ
Eγ
γ-/-
d
Rγ
Eγ
γ-/-

Figure 3.2 Specificity of the SK23 γ-synuclein antibody stain

(a) γ-synuclein staining in the midbrain of an adult wild-type mouse. Intense neuropil staining can be seen in the SN (arrow).

(b) The same antibody applied to a section taken from a γ-synuclein null mutant mouse illustrates the specificity of the affinity purified SK23 anti-γ-synuclein antibody, as no staining is seen above a very little background. The nigral region is marked (arrow). The lower panel is the adjacent section stained with an anti-TH primary antibody. The SN and VTA are clearly visible.

a



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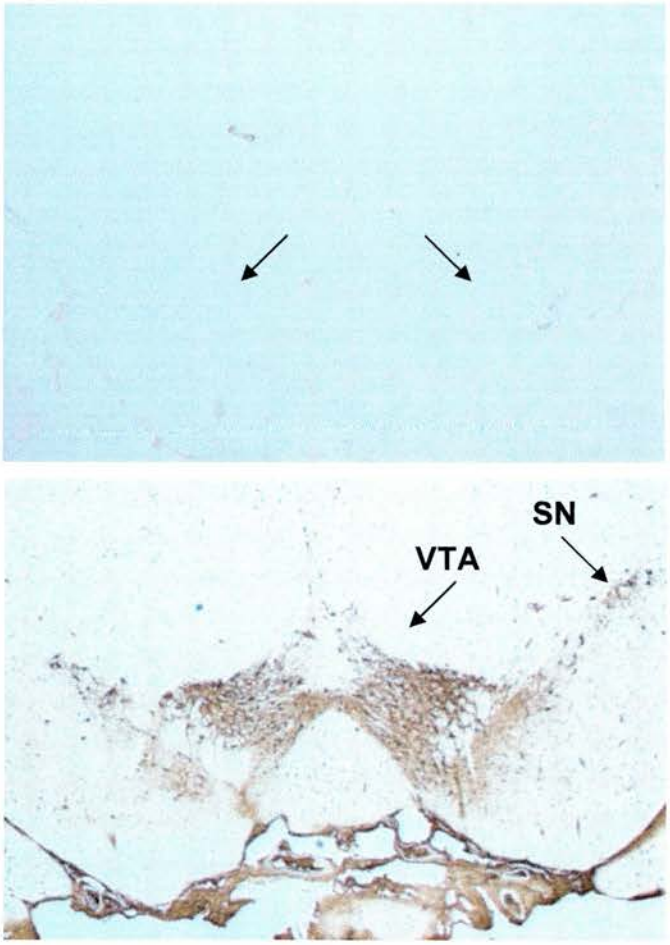


At E12 the TG neurones were found to be intensely stained for γ-synuclein, showing both axonal and cell body staining (Fig. 3.3a). This was also found to be the case at E15 (Fig. 3.3b) and P2, by which time neuronal populations have stabilised (Fig. 3.3c). Significantly, no changes were observed in the pattern of expression or in protein compartmentalisation during the studied period of development. All cells within the TG appear to express γ-synuclein rather than discrete populations of cells. In the DRG similar observations were made. At E12 γ-synuclein expression was observed in the perikarya and also in the nerve fibres of the dorsal root (Fig 3.4a). At E15 the expression pattern was unchanged, with axonal and cytoplasmic staining still clearly visible (Fig 3.4b). At P2 again the pattern was consistent with intense immunostaining in both the cytoplasm and nerve fibres of the DRG (Fig 3.4c).

3.3 Developmental changes in γ-synuclein compartmentalisation in sensory neurones

3.3.1 γ-synuclein mRNA expression

Expression of γ-synuclein mRNA and protein has been shown previously in sensory neuronal populations such as the trigeminal ganglion (TG) and the dorsal root ganglion (DRG) (Buchman et al., 1998). In the TG this expression begins at E10 and at E14 has reached a level which is maintained throughout subsequent development. The increase of expression level in TG between E10-12 coincides with the period of mouse embryonic development at which the earliest axons are growing to their target fields (Buchman et al., 1998).

It was necessary to verify that the protein expression does indeed match the mRNA expression pattern previously described (Buchman et al., 1998) If γ-synuclein is being expressed during this critical phase in development it may shed light on its physiological role. It was also important to study the sub-cellular localisation in addition to its presence or absence in certain structures.

3.3.2 γ-synuclein protein localisation in sensory neurones

An immunohistochemical investigation was performed using the highly specific affinity purified anti-γ-synuclein antibody to detect endogenous γ-synuclein on coronal and sagittal sections of mouse embryos at different developmental stages; thus allowing us to study any changes in protein localisation that may occur during development. This work was carried out in collaboration with Dr. Oliver Schmidt as described above (Chapter 2, section 2.6).

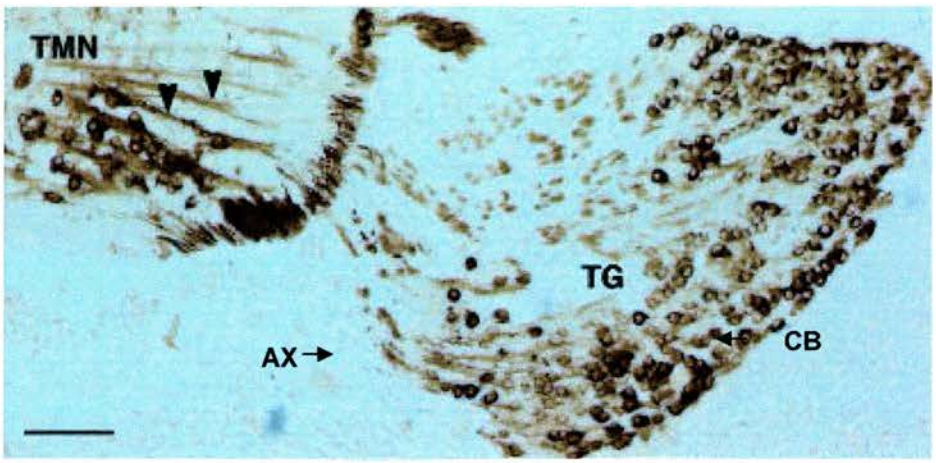
Figure 3.3 γ-synuclein expression in mouse trigeminal sensory neurones

(a) E12 mouse trigeminal motor nuclei (TMN) and the sensory trigeminal ganglia (TG) stained with affinity purified anti-γ-synuclein antibody showing the intensely positive cell bodies (CB) and axons (AX) of the TG in addition to those of the TMN. Arrowheads denote positively stained axons of the trigeminal motor nucleus. Scale bar represents 100μm.

(b) E15 mouse trigeminal motor nuclei (TMN) and the sensory TG stained with affinity purified anti-γ-synuclein antibody showing the intensely positive CB and axonal fibres (arrow) of the TG in addition to those of the TMN (arrowhead) and the root of the trigeminal nerve (rTN). Scale bar represents 200μm.

(c) Mouse P2 TG, CBs stained positive for γ-synuclein, in addition the AX staining of the trigeminal nerve (TN). Scale bar represents 100μm.

a



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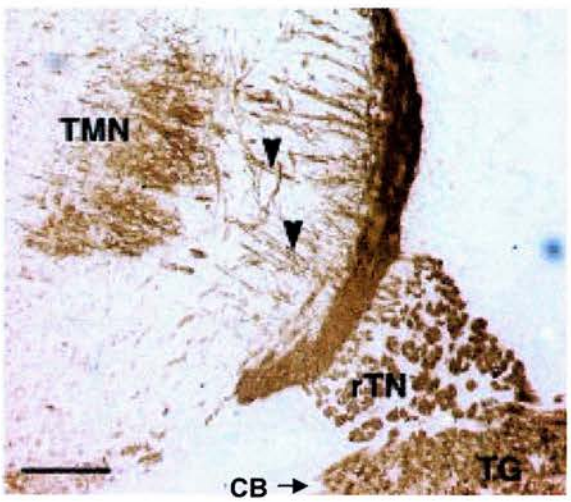


c

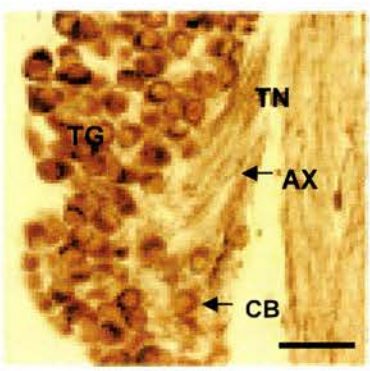


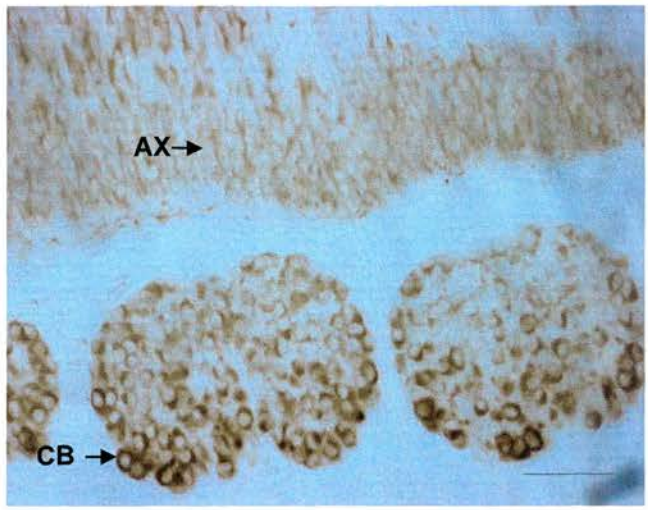
Figure 3.4 γ-synuclein expression in mouse dorsal root ganglion sensory neurones

(a) E12 mouse dorsal root ganglion (DRG), stained with affinity purified anti-γ-synuclein antibody showing the intensely positive cell bodies (CB) and axons. Scale bar represents 100μm.

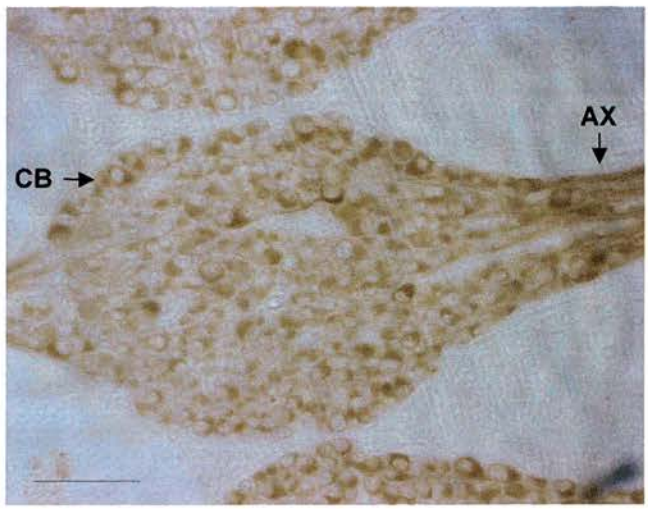
(b) E15 mouse DRG stained with affinity purified anti-γ-synuclein antibody showing the intensely positive CB and axonal fibres (AX) of the dorsal root. Scale bar represents 200μm.

(c) P2 mouse DRG CB staining positive for γ-synuclein. Positive staining of the fibres in the dorsal root (DR) is indicated. Scale bar represents 20μm

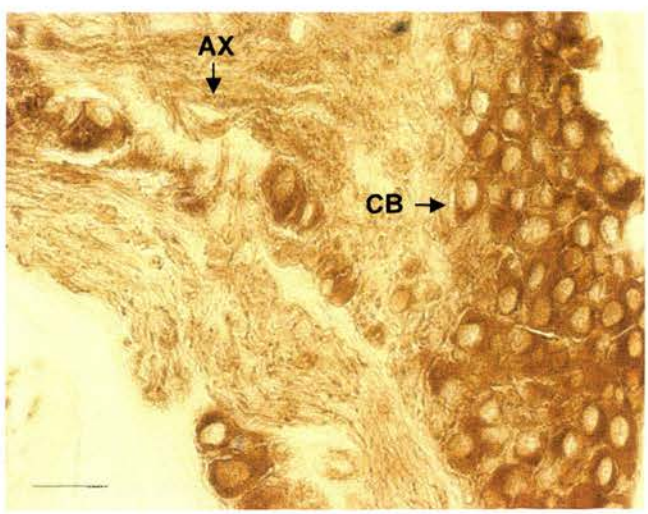
a



b



c



3.4 Developmental changes in intracellular compartmentalisation of γ-synuclein in *substantia nigra* neurones

3.4.1 γ-synuclein in the *substantia nigra*

Previous *in situ* hybridisation experiments have shown that γ-synuclein mRNA is expressed in the *substantia nigra* (SN) of adult mice (Kholodilov et al., 1999; Abeliovich et al., 2000). In addition to this, the neuropathological link of the synucleins to disorders impacting on the SN make it critical that protein expression be examined. To investigate γ-synuclein protein expression/distribution during development, the affinity purified anti-γ-synuclein antibody described above (section 3.2) was used for immunohistochemistry on coronal and sagittal paraffin sections of murine midbrain at different developmental stages. Anti-tyrosine hydroxylase antibodies were used to identify the dopaminergic neurones making up this structure (Chapter 2, section 2.7.4).

3.4.2 Embryonic expression of γ-synuclein in the *substantia nigra*

At E15 the dopaminergic cells making up the primordium of the SN begin to synthesise dopamine and hence have also produce tyrosine hydroxylase (TH). Thus, antibodies to TH were used to identify the SN and adjacent sections were stained for γ-synuclein (Chapter 1, section 1.6.2).

We have clearly demonstrated that at E15 γ-synuclein is expressed in both neuronal cell bodies and axons of the dopaminergic neurones making up the developing SN (Fig. 3.5a, b). Interestingly however three days later, at E18, the previously strong

neuronal cell body staining disappears, and is replaced with the dotted neuropil staining (Fig. 3.6a). The axonal staining however still remains prominent (Fig. 3.6b).

3.4.3 Postnatal expression of γ-synuclein in the *substantia nigra*

At P2 anatomical separation between functionally distinct *substantia nigra pars compacta* (SNpc) and ventral tegmental area (VTA) is possible. Drawing a boundary between the two structures was done arbitrarily as described above (Chapter 2, section 2.7.4). In both these structures, the diffuse pattern of neuropil staining persisted with no γ-synuclein immunoreactivity evident within the perikarya of the neuronal cells (Fig. 3.7a). However, other structures in the same region of the midbrain continued to display intense immunostaining with our affinity purified γ-synuclein antibody. Such structures included the red nucleus which displays prominent immunostaining in the perikarya (Fig. 3.7). Also visible were the nerve fibres of the medial lemniscus (Fig. 3.7).

3.4.4 Expression of γ-synuclein in the adult *substantia nigra*

The pattern of γ-synuclein expression was studied in adult animals over four months old using identical methods to those described above. The SNpc revealed an irregular pattern of cells staining positive for γ-synuclein. These cells were distributed in a haphazard fashion and numbers varied from animal to animal. No such cells were observed in the VTA. To prove that the γ-synuclein positive cell was truly a dopaminergic neurone of the SNpc, double immunofluorescence detection of two proteins on the same section was performed (Fig. 3.8a, b). The affinity purified rabbit polyclonal anti-γ-synuclein antibody was used in conjunction with a TRITC-

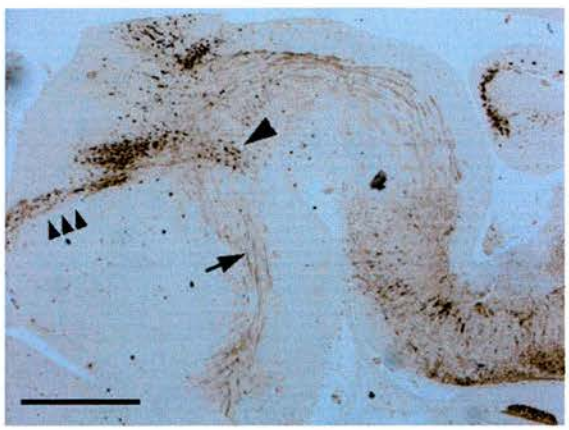
conjugated secondary anti-rabbit antibody and mouse monoclonal anti-TH antibody in conjunction with a FITC-conjugated secondary anti-mouse antibody. In the postnatal brain, punctate neuropil staining was detected in the striatum indicating γ-synuclein may not only be located in axons but at presynaptic regions of the dopaminergic neurones of the SNpc (Fig. 3.9).

Figure 3.5 Sagittal section of E15 mouse brain showing the primordium of the substantia *nigra stained* with anti-γ-synuclein antibody

(a) Sagittal section showing the primordium of the SN in E15 mouse brain. The three arrowheads denote the fasciculus retroflexus, a critical landmark in positive identification of the nigra. The arrow indicates the nigro-striatal tract, while the arrowhead identifies the primordium of the SN positively stained for γ-synuclein. Scale bar represents 500μm.

(b) Individual positively stained cell bodies of the primordial SN at higher magnification. Scale bar represents 20μm.

a



b

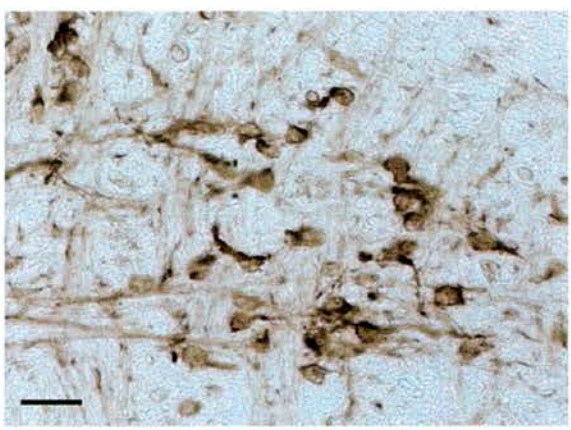
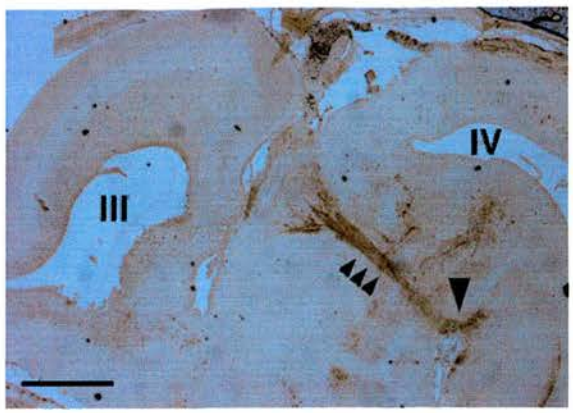


Figure 3.6 Sagittal section of E18 mouse brain showing the distribution pattern of γ-synuclein in the primordial *substantia nigra*

(a) The three arrowheads denote the fasciculus retroflexus, in addition the landmarks that are the third (III) and fourth (IV) ventricles are also highlighted. The large arrow indicates positively stained cells in the SN. Scale bar represents 500μm.

(b) The SN is shown at higher magnification but in contrast to that seen at E15, at E18 cell body staining has been replaced with dotted neuropil staining identifying the nigra. Scale bar represents 50μm.

a



b

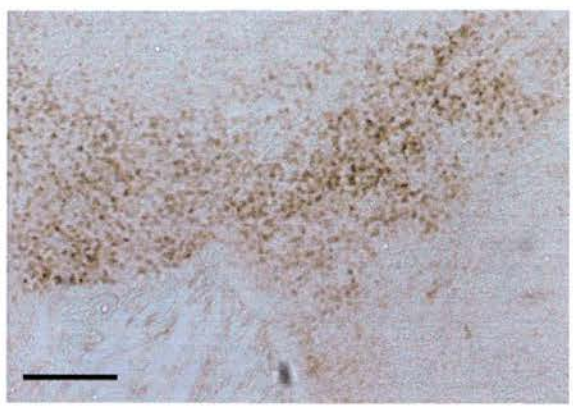
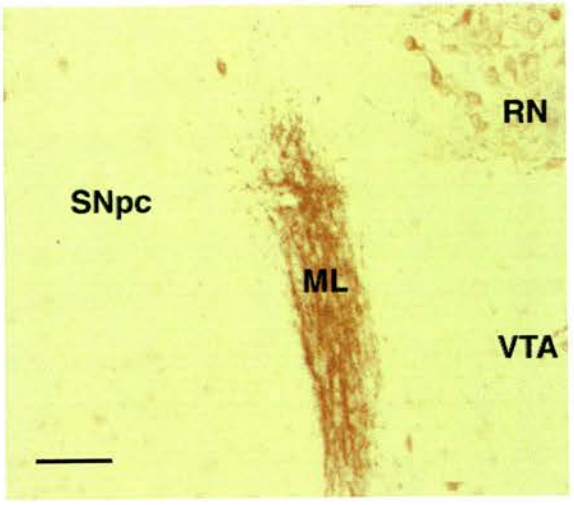


Figure 3.7 Coronal section of P2 mouse brain at the level of the *substantia nigra*

(a) Shows the region of the *substantia nigra* stained with anti-γ-synuclein antibody. The cell bodies of the neurones of the red nucleus (RN) and the fibres of the medial lemniscus (ML) can be seen to stain positive for γ-synuclein, providing a positive internal control as well as a landmark. Note the lack of cell body staining within the *substantia nigra pars compacta* (SNpc) and the ventral tegmental area (VTA). (Scale bar 50μm).

(b) Shows the consecutive paraffin section to that shown in (a) stained with anti-TH antibody to delineate the SNpc and VTA. (Scale bar 200μm).

a



b

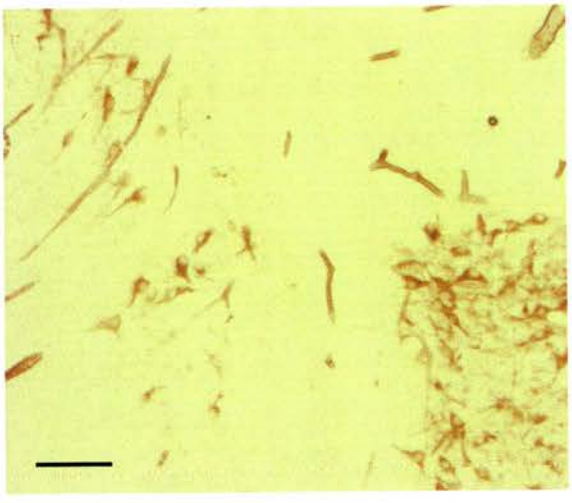
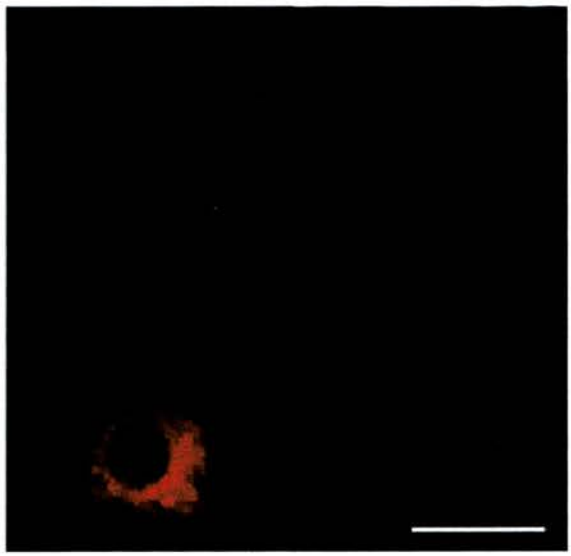


Figure 3.8 Dopaminergic cells of the adult SNpc express γ-synuclein

(a) A SNpc neurone stained with rabbit polyclonal anti-γ-synuclein antibody with a TRITC- conjugated secondary anti-rabbit antibody.

(b) SNpc neurones stained with mouse monoclonal anti-TH antibody with FITC-conjugated secondary anti-mouse antibody.

a



b

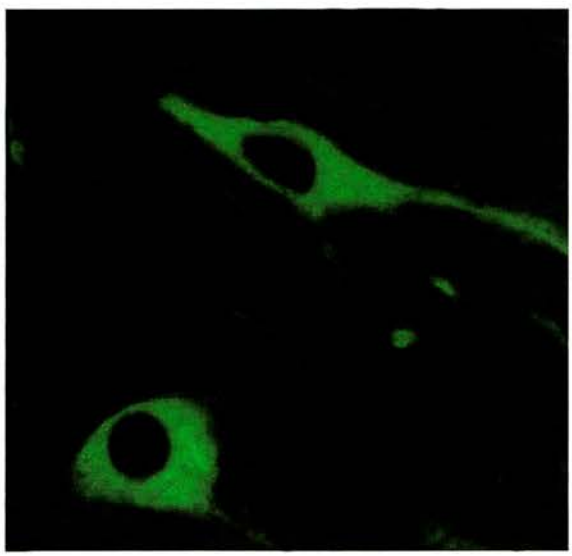
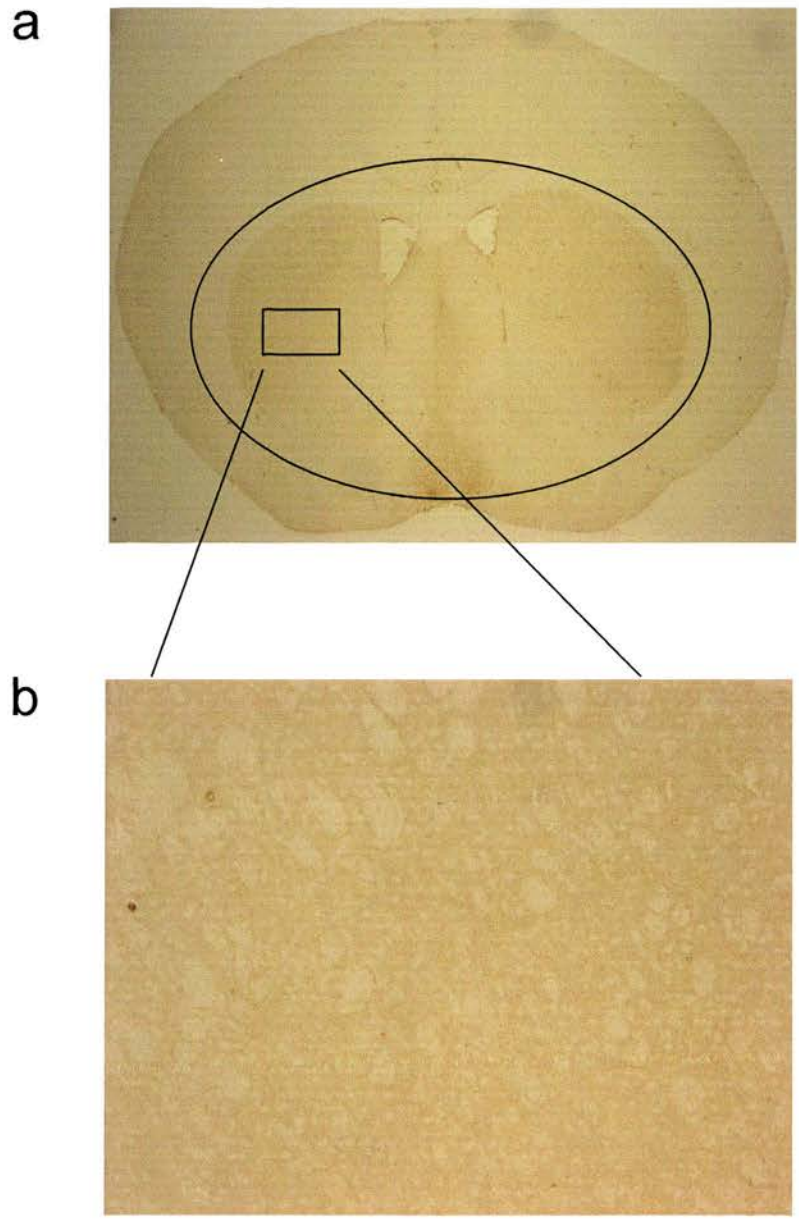


Figure 3.9 γ-synuclein expression in the adult striatum

(a) The striatum of a wild-type adult mouse stained with the affinity purified γ-synuclein antibody. Note the neuropil appearance of the stain surrounded by the black oval. Neuropil staining is defined as the complex net of axonal, dendritic, and glial branching that forms the bulk of the central nervous system grey matter.

(b) A higher power image of γ-synuclein neuropil staining in the striatum. This staining is specific and above background.


a
b

3.5 The generation and maintenance of a $\gamma^{-/-}$ colony

3.5.1 Chimera generation

Production of the first chimeric animal carrying the mutated-synuclein allele was successfully completed and is described previously (Chapter 1, section 1.8.1). The initial crossing of a male chimera with wild-type C57Bl6 females produced the F1 generation of animals with brown coat colour, a sign of successful germline transfer of the ES cell genotype in the chimera. Theoretically, a proportion (50%) of these animals should have been heterozygous for the mutated γ-synuclein allele. Selection of animals carrying this mutation for further use was based on results of genotyping using polymerase chain reaction (PCR) with specifically designed primers (Chapter 2, section 2.3.6).

3.5.2 Genotypic verification

Establishing a reliable and efficient method of genotyping animals was the most critical step in producing experimental null mutant animals. Primers were designed using appropriate sequences from both the wild-type and mutant alleles, and the optimal PCR reaction was established by Dr. N. Ninkina before this project started. DNA was extracted from ear or tail biopsies for use in genotyping reactions (Chapter 2, section 2.3.5). Established protocols for PCR amplification and subsequent agarose gel analysis of the reaction products, detected a 490bp fragment in the presence of the wild-type γ-synuclein allele and a 379bp fragment in the presence of the null mutant allele. The presence of both these fragments indicated a heterozygous animal (Fig. 3.10).

3.5.3 Purification of genetic background

The initial chimera was the product of a 3.5 day old C57Bl6 blastocyst injected with genetically modified 129Ola ES cells. Germline transfer therefore only occurred if gamete-producing cells of a chimera were formed from these 129Ola cells. A male chimera could mate with a wild-type female C57Bl6 and if the resulting litter had a mottled coat it indicated successful germline transfer. The resulting pups however were on a mixed genetic background. Different mouse strains such as C57Bl6 and 129Ola have been shown to have very different behavioural, physiological and even morphological features. The inter-strain differences are not predictable or regulated in offspring, hence any investigation into behaviour, physiology or morphology would be flawed and any data gleaned inaccurate. To overcome this, the colony was backcrossed in order to ensure that all experimental animals would be on a pure genetic background. This was achieved by mating heterozygous male animals with wild-type C57Bl6 female animals (Fig. 3.11a). This process was repeated until the F6 generation was reached, the minimum level on which behavioural and morphometric experiments were carried out.

3.5.4 Experimental animals

When the F6 generation was reached, experimental animals were generated. This was achieved by inter-crossing heterozygous male and female littermates in order to produce a heterogeneous litter. This litter should contain wild-type ($\gamma^{+/+}$), null mutant ($\gamma^{-/-}$) and heterozygous ($\gamma^{+/-}$) animals, all of F6 generation (Fig. 3.11b). Genotyping, as described above using PCR amplification (Chapter 2, section 2.3.6), revealed that

animals were born in normal Mendelian ratios indicating that $\gamma^{-/-}$ animals were equally as viable as $\gamma^{+/-}$ or $\gamma^{+/+}$ animals.

3.5.5 Proving absence of γ-synuclein mRNA and protein

Genotyping conclusively showed that both alleles were mutated in the first $\gamma^{-/-}$ animals. To ensure that the introduced mutation resulted in complete inactivation of the gene, we compared γ-synuclein mRNA expression in retinas of $\gamma^{-/-}$ and $\gamma^{+/+}$ animals. This tissue was chosen because expression of all three synucleins has been demonstrated previously in the retina (Surguchov, McMahan, Masliah and Surgucheva, 2001). Therefore any compensatory changes in the expression of the remaining synucleins could be assessed simultaneously. $\gamma^{-/-}$ and $\gamma^{+/+}$ animals were sacrificed by appropriate Schedule 1 method, retinas were dissected and total mRNA extracted and separated on a 1.2% agarose gel then transferred onto a nylon membrane (Chapter 2, section 2.4.1).

Northern hybridisation was performed, using a highly specific full length γ-synuclein cDNA probe (Chapter 2, section 2.4.4). Figure 3.12(a) clearly shows the complete absence of γ-synuclein mRNA in retina of $\gamma^{-/-}$ mice as compared directly with wild-type retina showing definite expression.

Under the hybridisation and moderate stringency washing conditions the γ-synuclein cDNA probe cross-hybridised with the β-synuclein transcript, producing an aberrant band (Fig. 3.12a*). High stringency washes completely eradicated this leaving the γ-synuclein hybridisation signal in wild-type samples unchanged (Fig. 3.12a).

The same blot was stripped and re-hybridised with a specific 3' UTR probe accurately designed to exclusively detect β-synuclein mRNA. This band coincided

with an aberrant band detected when probing for γ-synuclein, hence was identified as a β-synuclein cross-hybridisation. No changes were detected in the level of β-synuclein mRNA in $\gamma^{-/-}$ (Fig 3.12a).

Once again the blot was stripped after the β-synuclein hybridisation and re-probed with an α-synuclein specific 3' UTR probe, and again no changes were seen in the level of α-synuclein mRNA expression in $\gamma^{-/-}$ mice (Fig 3.12a).

Finally the blot was striped once more and re-hybridised with a specific probe for the housekeeping gene GAPDH, which ensured equivalent levels of total RNA in each sample were present on the filter (Fig. 3.12a).

The absence of γ-synuclein protein in tissues from the null mutants was confirmed by Western blotting as described above (Chapter 2, section 2.5). Spinal cord tissue was dissected from adult $\gamma^{-/-}$ and $\gamma^{+/+}$ mice. Tissue was homogenised in loading buffer, total protein concentration in homogenate was measured and equal amounts were loaded onto a 15% polyacrylamide gel. Separated proteins were transferred onto a PVDF membrane and probed with the affinity purified, highly specific γ-synuclein antibody described above. The result shown in Fig. 3.12(b) confirmed that no γ-synuclein protein was present in the $\gamma^{-/-}$ mice.

Figure 3.10 PCR analysis of genotype

Image of a typical agarose gel illustrating the genotyping of animals from an intercross litter from the $\gamma^{-/-}$ colony. The presence of both $\gamma^{+/+}$ 490bp band and $\gamma^{-/-}$ 397bp mutant allele band can be seen indicating the presence of two heterozygotes ($\gamma^{+/-}$), a $\gamma^{+/+}$ and a $\gamma^{-/-}$ animal.

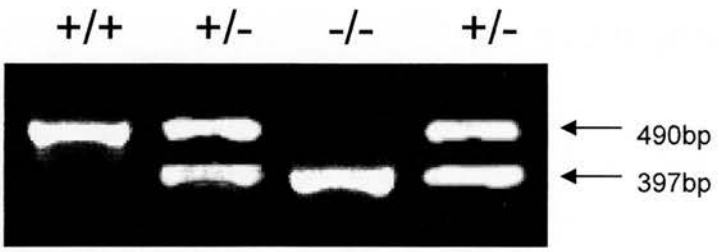
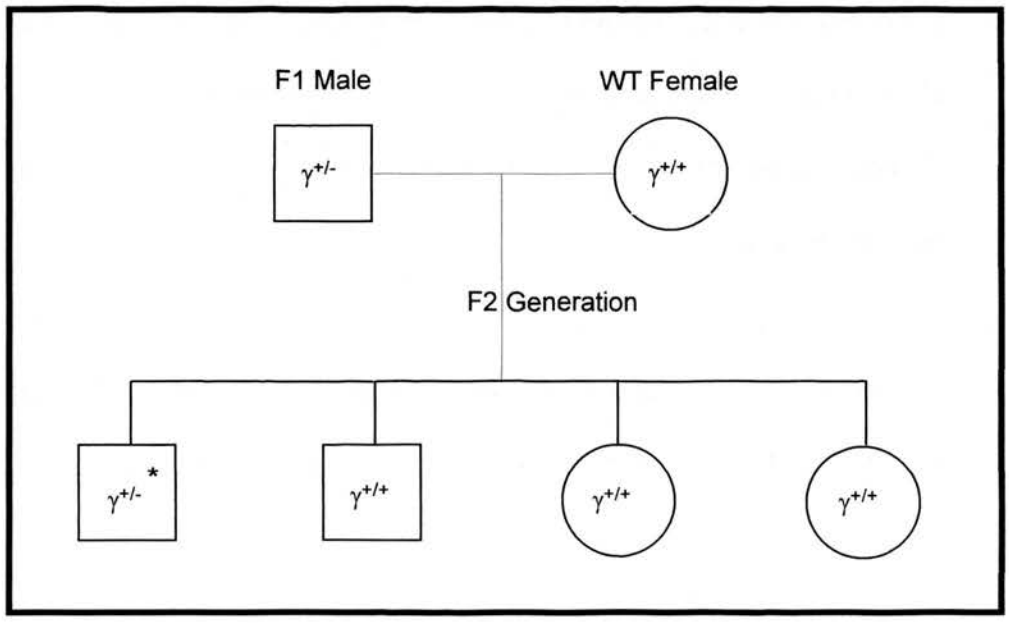

+/+
+/-
-/-
+/-
490bp
397bp

Figure 3.11 Diagram illustrating the process of transfer of the γ-synuclein transgenic colony onto a pure genetic (C57Bl6) background

(a) Illustrates the process of genetic purification of the γ-synuclein colony. Heterozygous male animals are mated with standard C57Bl6 females from Charles River. (*) denotes a male animal which can be used to produce the next generation.

(b) Illustrates the production of an experimental cohort of littermates, for experimental use, once F6 had been reached. The intercrossed litter retain the same F6 status.

a


F1 Male
WT Female
γ+/-
γ+/+
F2 Generation
γ+/-*
γ+/+
γ+/+
γ+/+

b

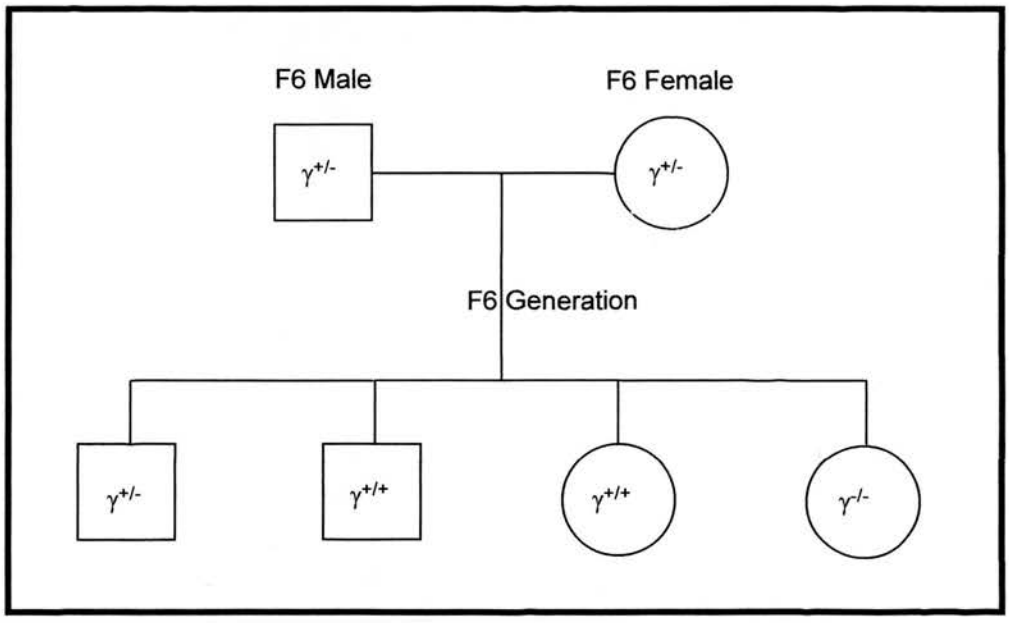
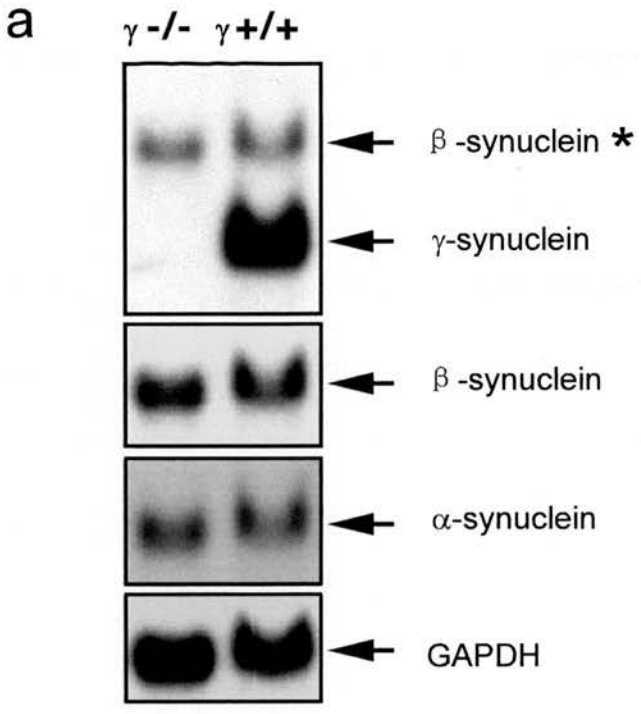
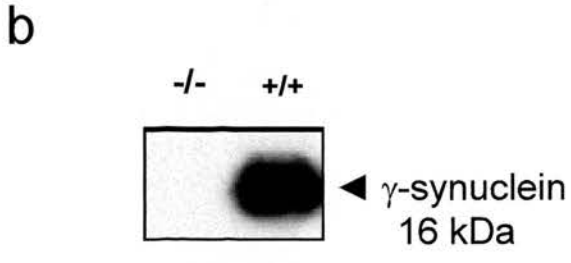

F6 Male
F6 Female
γ+/-
γ+/-
F6 Generation
γ+/-
γ+/+
γ+/+
γ-/-

Figure 3.12 Expression of synucleins in adult mice by Northern and Western blot

(a) Expression of mRNAs encoding members of the synuclein family in the retinas of $\gamma^{+/+}$ and $\gamma^{-/-}$. Results of Northern hybridisation with a full-length mouse γ-synuclein cDNA probe, a mouse β-synuclein-specific probe, a mouse α-synuclein-specific probe, and a GAPDH probe are shown. * indicates that under the hybridisation and washing conditions used, the γ-synuclein cDNA probe cross-hybridised with β-synuclein transcript. High-stringency washes completely eradicated this hybridisation signal, with no effect on hybridisation with γ-synuclein transcript.

(b) Western blot with 10 µg of total spinal cord protein lysates per lane was probed with an anti-γ-synuclein. Wild-type (+/+) sample shows high levels of γ-synuclein protein, clearly absent in the adjacent $\gamma^{-/-}$ sample.


a
γ -/-
γ +/+
β -synuclein *
γ-synuclein
β -synuclein
α-synuclein
GAPDH


b
-/-
+/+
γ-synuclein
16 kDa

3.5.6 Production of experimental α-synuclein null mutant mice

During the course of our investigations α-synuclein null mutant mice were obtained from the laboratory of A. Rosenthal (Genentech). These mice have been described in detail previously (Abeliovich et al., 2000). The mice were on a mixed 129Ola/C57Bl6 genetic background and hence were backcrossed as described above (Chapter 2, section 2.3.2). This ensured that all experimental animals were on the same genetic background, allowing for accurate comparisons to be made between the different genotypes, in this case $\alpha^{-/-}$ and $\gamma^{-/-}$.

3.5.7 Production of experimental $\alpha^{-/-}/\gamma^{-/-}$ double null mutant mice

Once both $\gamma^{-/-}$ and $\alpha^{-/-}$ mutant mice had been backcrossed to equivalent genetic backgrounds suitable for intercrossing (F6), it was feasible to create an $\alpha^{-/-}/\gamma^{-/-}$ synuclein double null mutant strain (Chapter 2, section 2.3.3). This colony would allow us to compare the cumulative effect of the null mutations alongside the single mutant animals on an identical genetic background.

Interbreeding $\alpha^{-/-}$ and $\gamma^{-/-}$ animals produced a litter of double heterozygous animals, in effect $\alpha^{+/-}$ / $\gamma^{+/-}$ animals. Interbreeding these offspring yielded a 1:16 chance of a litter containing a double null mutant mouse. These $\alpha^{-/-}$/ $\gamma^{-/-}$ mice could then be interbred to enlarge the colony.

3.6 Initial characterisation of γ-synuclein null mutant mice

3.6.1 γ-synuclein null mutant mice

Identification of the first $\gamma^{-/-}$ mouse in an intercross litter indicated this mutation was non-lethal during gestation. As further litters were produced, it became obvious that development was not restricted, as $\gamma^{-/-}$ animals appeared at approximately the expected mendelian frequency. $\gamma^{-/-}$ mice developed normally and phenotypically it was not possible to distinguish them from $\gamma^{+/+}$ and $\gamma^{+/-}$ littermates. A study of the gross morphology of the brain and other organs carried out by Dr. O. Schmidt also failed to reveal obvious differences between $\gamma^{+/+}$ and $\gamma^{-/-}$ mice. Male and female $\gamma^{-/-}$ animals were interbred and regular size litters of $\gamma^{-/-}$ mice were successfully produced proving $\gamma^{-/-}$ animals were fertile as well as viable. The average weight of $\gamma^{-/-}$ juvenile and adult animals was no different from their $\gamma^{+/+}$ and $\gamma^{+/-}$ littermates. This is illustrated by the comparison of 4 month old male animals (Fig. 3.13).

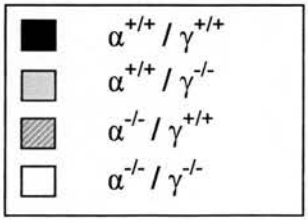
3.6.2 General level of activity displayed by $\gamma^{-/-}$ mice

To assess the level of general activity displayed by $\gamma^{-/-}$ in comparison with $\gamma^{+/+}$ mice an open field camera (Ugo Basile) was used. The average number of infrared beam breaks was recorded at 10 minute intervals over a 2 hour test period. Adult male animals were used, as hormonal changes in female mice can alter behaviour patterns, especially significant in a new environment such as the camera.

Studies of these animals in an open field camera revealed no significant differences in the general levels of activity between $\gamma^{-/-}$ and $\gamma^{+/+}$ mice (Fig. 3.14).

Figure 3.13 Average weight of 4 month old male of γ-, α- or α/γ-synuclein null mutant mice compared to wild-type mice

The average weight of 4 month old male γ-, α- or γ/α - synuclein null mutant mice was compared to wild-type mice. No statistically significant differences were observed. n=8 for each genotype.



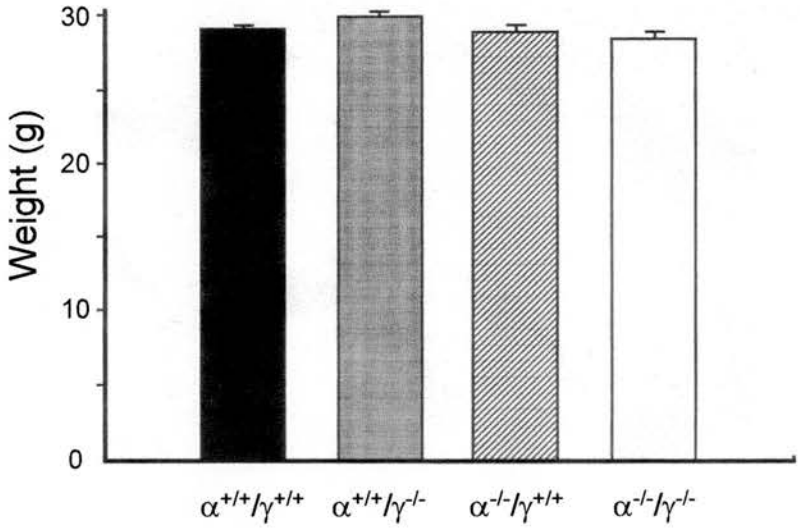
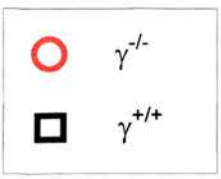
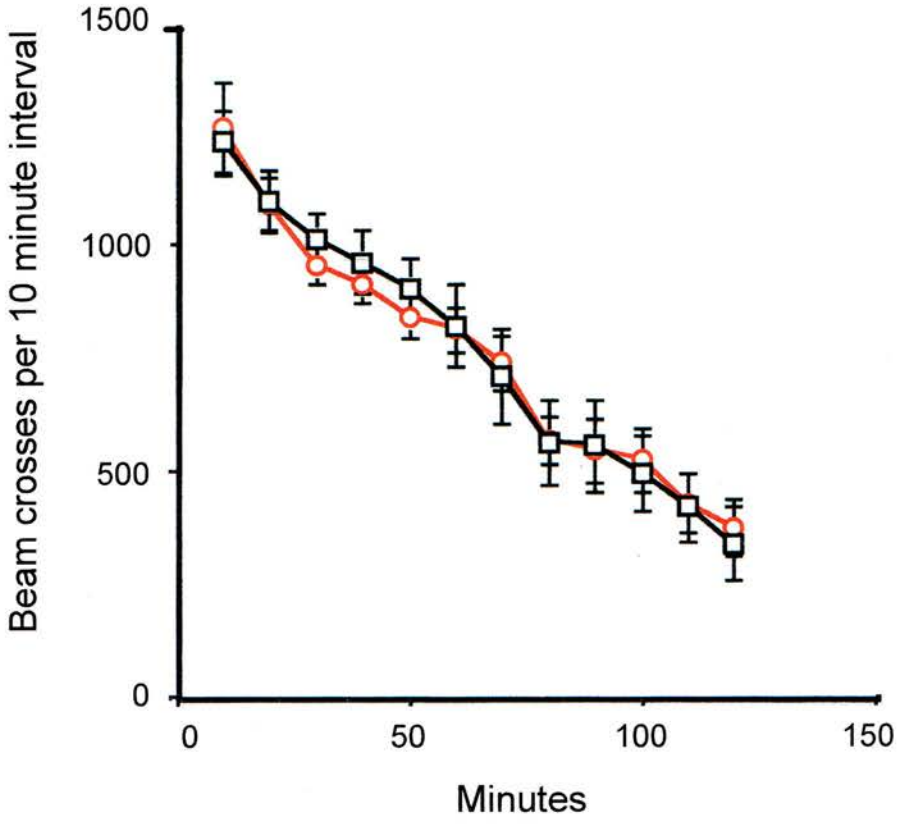

Weight (g)
30
20
10
0
α+/+/γ+/+
α+/+/γ-/-
α-/-/γ+/+
α-/-/γ-/-

Figure 3.14 Assessment of general activity using an open field camera

The general level of activity of γ-synuclein null mutant mice ($\gamma^{-/-}$) was tested in comparison with that of wild-type ($\gamma^{+/+}$) littermates. The mean number of beam breaks in a given time was recorded and no significant differences were observed. n=6 for each group.




1500
1000
500
0
Beam crosses per 10 minute interval
0
50
100
150
Minutes

3.7 Neuronal number of dorsal root ganglion (DRG) and the trigeminal ganglion (TG) of $\gamma^{+/+}$, $\gamma^{-/-}$ and $\alpha^{-/-}$ animals

3.7.1 γ-synuclein in sensory ganglia

We have shown that γ-synuclein expression begins during embryonic development and is maintained throughout the postnatal period into adulthood. In contrast to γ-synuclein, α-synuclein has been shown to be radically different, being expressed heterogeneously throughout the CNS with immuno-positive structures being punctuate with a neuropil appearance (Totterdell et al., 2004). Expression however begins at approximately the same point during development, E12 (Hsu et al., 1998). Peripheral sensory neurones display one of the highest levels of γ-synuclein expression in the entire nervous system (Buchman et al., 1998). Therefore in the absence of any gross anatomical or behavioural changes in $\gamma^{-/-}$, it was logical to investigate any effects of this loss in an area of high expression. At P2 the period of natural cell death (NCD) has ended and the neuronal population stabilised both in the DRG and TG, therefore this is a suitable time point for analysis (Chapter 1, section 1.6.3). α-synuclein is also expressed in these neurones, although at a lower level. Therefore α-synuclein null mutants were also included in the study. Double $\alpha^{-/-}/\gamma^{-/-}$ were not available at the time of this experiment hence not included in the study.

3.7.2 Assaying sensory neuronal populations

Neuronal populations of the trigeminal ganglia (TG) and L6 dorsal root ganglia (DRG) were assayed in P2 mice. Tissues were dissected and processed for histology as described above (Chapter 2, section 2.6) Longitudinal sections of the lumbar region of the spinal cord and transverse sections of the head were stained with Cresyl

Fast Violet to facilitate the identification and assessment of these neuronal populations (Chapter 2, section 2.8.1).

Cell counts were carried out using a stereology system as described previously (Chapter 2, section 2.8.2).

No statistically significant differences were found in the mean number of neurones per ganglia in the TG of wild-type, $\gamma^{-/-}$ and $\alpha^{-/-}$ mice, with all genotypes containing approximately 30,000 neurones per ganglion (Fig 3.15a).

Identical results were observed in the L6 DRG, where again no statistically significant differences were observed in the mean number of neurones per ganglion in the DRG of wild-type, $\gamma^{-/-}$ and $\alpha^{-/-}$ mice, all having approximately 2,500 neurones per ganglion (Fig. 3.15b). These results clearly indicate that the absence of either α- or γ- synuclein does not have any direct effect on the development of sensory neuronal populations of either the TG or DRG.

Figure 3.15 Sensory neuronal number assayed in the trigeminal and dorsal root ganglia of wild-type ($\alpha^{+/+}/\gamma^{+/+}$), γ-synuclein null ($\gamma^{-/-}$) and α-synuclein ($\alpha^{-/-}$) null mutant mice

(a) Comparison of the mean (± SEM) number of neurones in the TG of $\alpha^{+/+}/\gamma^{+/+}$, $\gamma^{-/-}$ and $\alpha^{-/-}$ P2 mice. Means were taken from at least 10 ganglia for each genotype. There were no significant differences in the neuronal number irrespective of genotype ($p > 0.6$, Kruskal-Wallis one-way ANOVA analysis of variance).

(b) Comparison of the mean (± SEM) number of neurones in the L6 DRG of $\alpha^{+/+}/\gamma^{+/+}$, $\gamma^{-/-}$ and $\alpha^{-/-}$ P2 mice. Means were taken from at least 10 ganglia for each genotype. There was no significant difference in the neuronal number irrespective of genotype ($p > 0.6$, Kruskal-Wallis one-way ANOVA analysis of variance).

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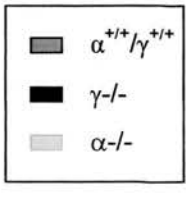
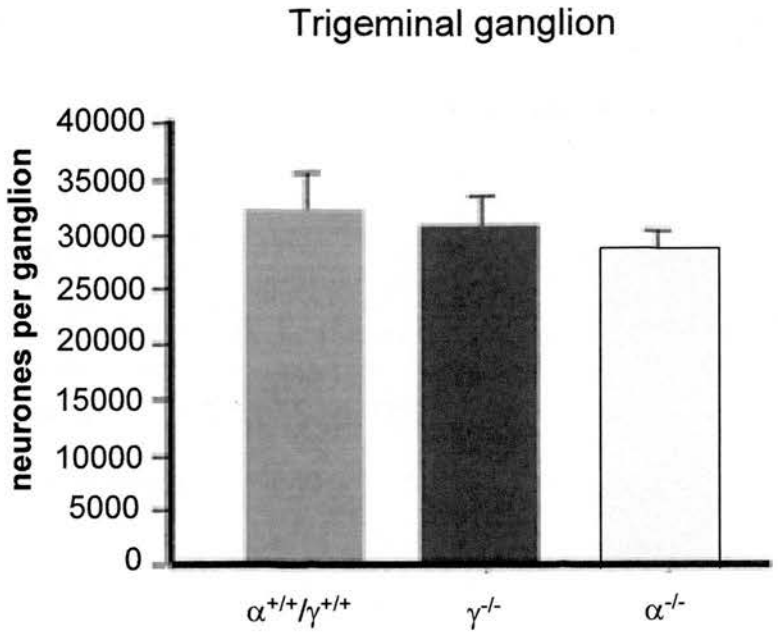
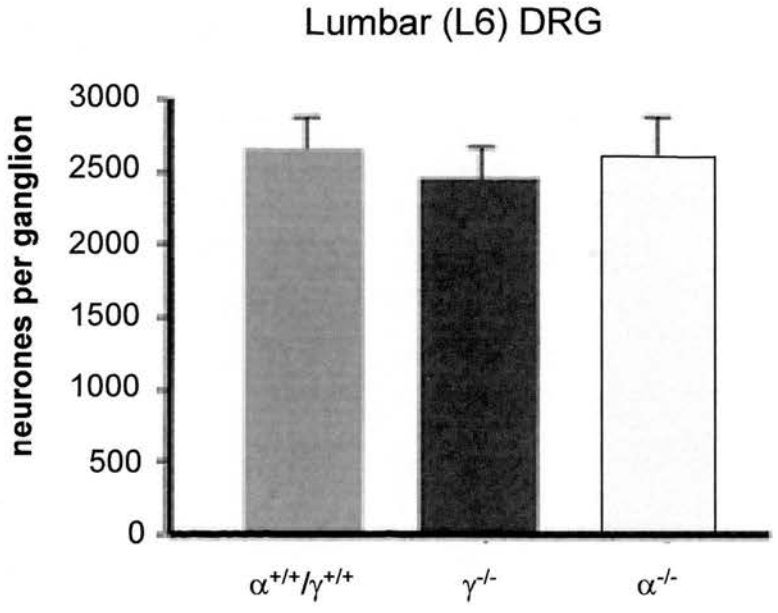


a


Trigeminal ganglion
neurones per ganglion
40000
35000
30000
25000
20000
15000
10000
5000
0
α+/+/γ+/+
γ-/-
α-/-

b


Lumbar (L6) DRG
neurones per ganglion
3000
2500
2000
1500
1000
500
0
α+/+/γ+/+
γ-/-
α-/-

3.8 Survival in culture of peripheral nervous system neurones of wild-type and synuclein null mutant mice

3.8.1 Primary neuronal cultures

Although no differences in neurone numbers in DRG or TG of wild-type, α-synuclein or $\gamma^{-/-}$ were found, it was feasible to check whether neurones with an incorrect balance of synucleins are more sensitive to various stresses than wild-type neurones. For this we compared the survival of neurones from the trigeminal ganglia of $\alpha^{-/-}$ and $\gamma^{-/-}$ and $\alpha^{-/-}/\gamma^{-/-}$ mice in dissociated primary cultures. It has been shown previously that certain types of cells with modified expression of synucleins, particularly dopaminergic neurones over-expressing α-synuclein, have normal survival characteristics under optimal culture conditions but are substantially more susceptible to stresses (Ostrerova-Golts, Petrucelli, Hardy, Lee, Farer and Wolozin, 2000; Petrucelli et al., 2002).

To determine whether the absence of γ-synuclein renders neurones more sensitive to various toxic insults, we cultivated P2 trigeminal neurones in the absence of antioxidants or in the presence of DNA-damaging agents, proteasome inhibitors, heavy metal ions or inhibitors of major intracellular signalling pathways in the medium. Cultures from trigeminal ganglia were prepared and maintained in neurobasal medium with B27 complement as described above (Chapter 2, section 2.12). The number of neurones attached to each culture dish within a 12 by 12mm square was counted 3 hours after plating and was taken as the initial number of neurones, the 100% value. In all cases, drugs were added to cultures immediately after the initial count. The number of surviving neurones in the same area was counted at 48 hours and was expressed as a percentage of the initial count.

3.8.2 Neuronal survival in dissociated culture

Firstly, the survival characteristics of α-synuclein and γ-synuclein deficient neurones were studied in the presence and absence of nerve growth factor (NGF). In both cases, the survival of these neurones was indistinguishable from those of wild-type neurones. They survived similarly well in the presence of NGF and failed to survive equally in its absence (Fig. 3.16a).

A selection of pharmaceutical agents were chosen to apply to cultures on the basis of their known neurotoxicity or because of putative interactions of α-synuclein and proteins involved in certain signalling pathways discussed in greater details above (Chapter 1, section 1.3).

In the presence of the proteasome inhibitors MG-132 and PSI and the heavy metal ions Cu^{2+} and Zn^{2+} no significant differences in survival were observed in the absence of γ-synuclein; equal reductions were observed for both genotypes alike, in the presence of these neurotoxic agents (Fig. 3.16b).

In the absence of antioxidants or the presence of the cytotoxic agents AraC and etopicide equivalent observations were made i.e. no significant differences in survival were observed in the absence of γ-synuclein (Fig. 3.16c).

Finally in the presence of inhibitors of the JNK signalling pathway SP600125, ERK signalling pathway PD98059, or phosphatidylinositol 3-kinase signalling pathway LY294002 no statistically significant differences in survival were observed in the absence of γ-synuclein (Fig. 3.16d.)

Figure 3.16 Survival of P2 mouse trigeminal ganglion neurones in dissociated primary culture

Bar charts illustrate survival of neurones 48 hours after initial count and addition of drugs. The number of surviving neurones is expressed as a percentage of the initial count. Means ± SEM of data obtained from analysis of at least six culture dishes for each genotype in two independent experiments are shown.

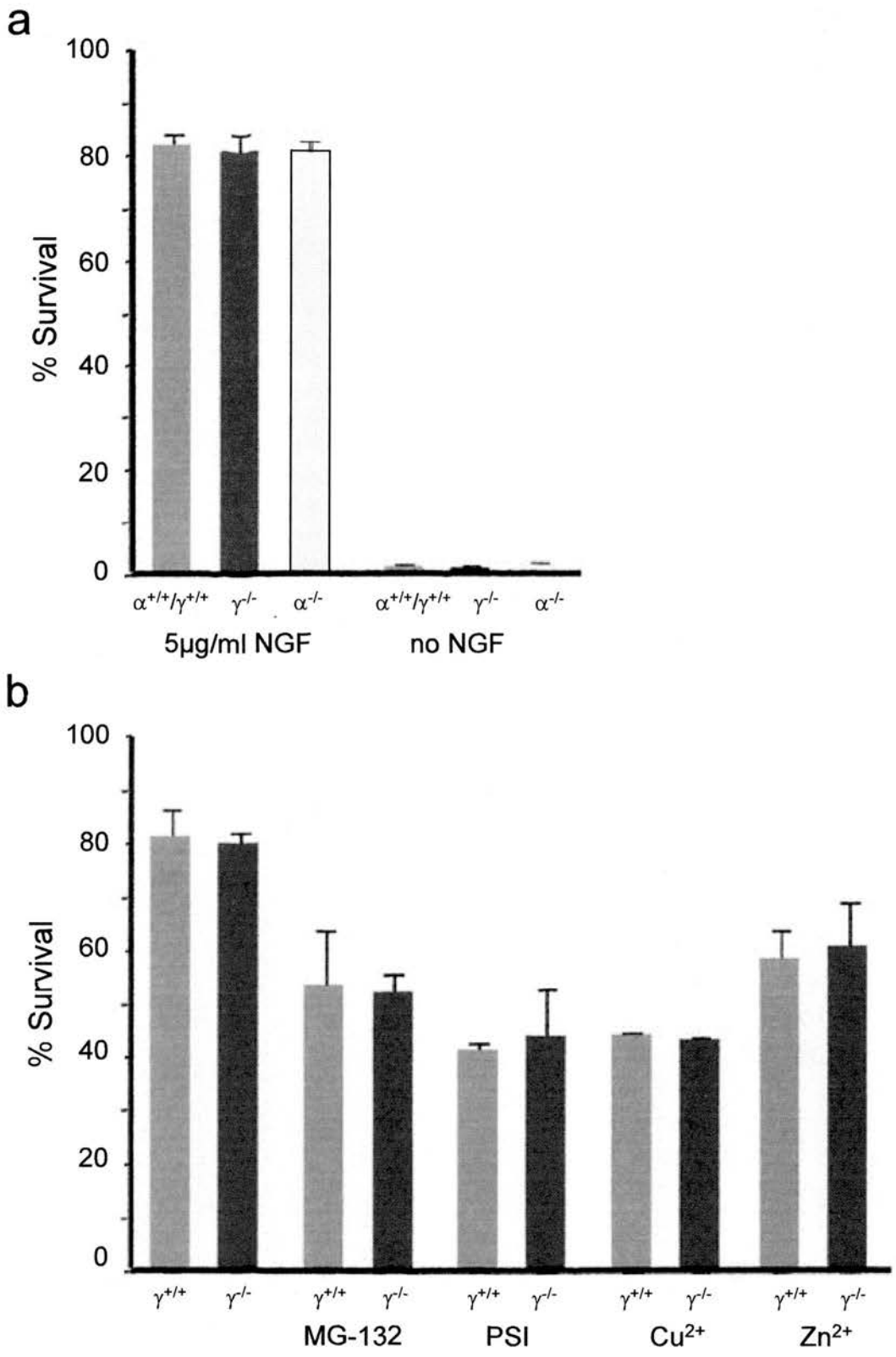
(a) Both α-synuclein deficient ($\alpha^{-/-}$) and γ-synuclein deficient ($\gamma^{-/-}$) neurones have the same survival rate as wild-type ($\alpha^{+/+}/\gamma^{+/+}$) neurones in the presence of nerve growth factor (NGF) and are unable to survive in its absence ($P < 0.8$, Kruskal-Wallis one-way ANOVA).

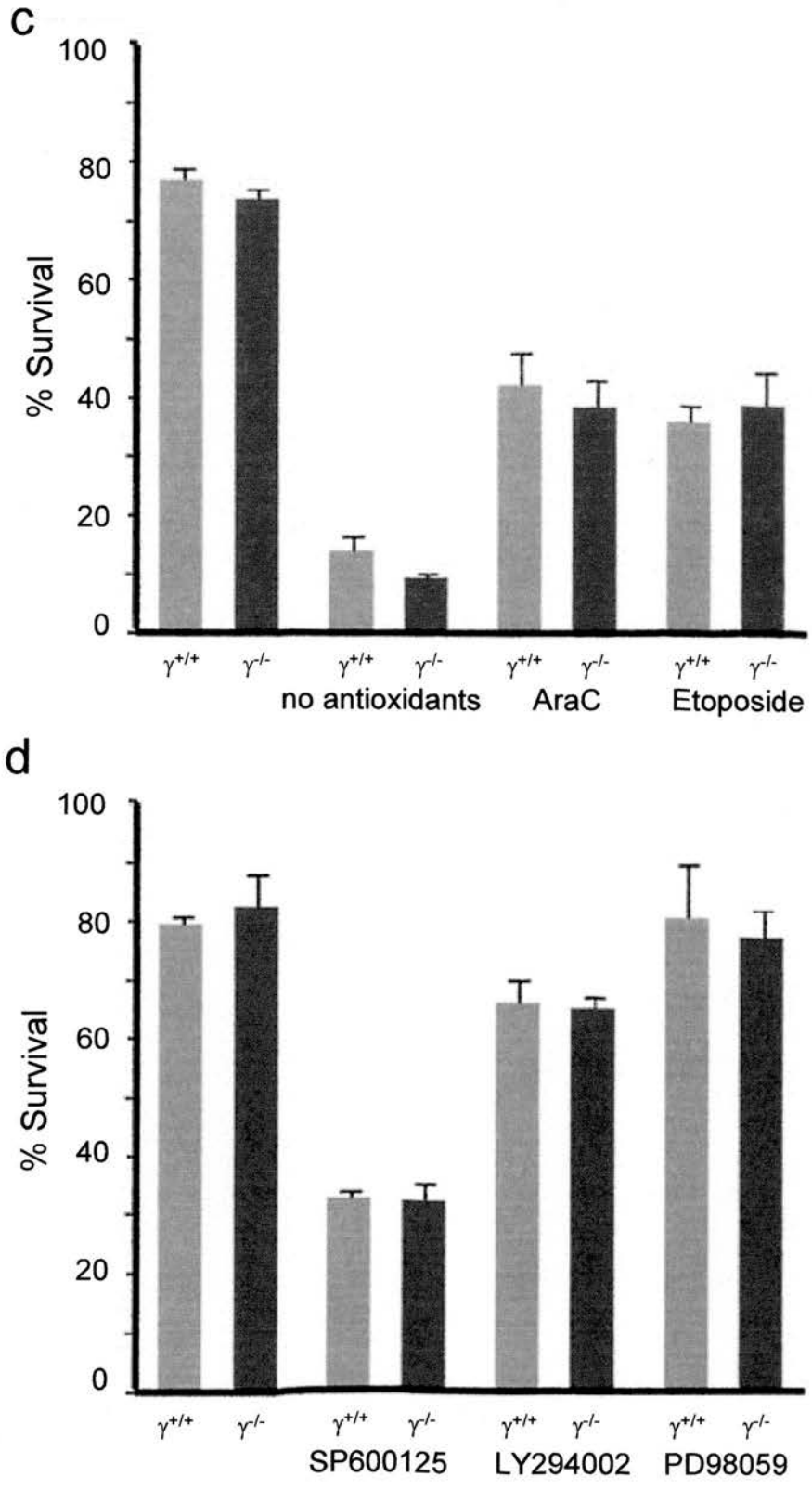
(b) Proteasome inhibitors MG-132 or proteosome inhibitor I [PSI]) and heavy metal ions Cu^{2+} or Zn^{2+} were added to cultures after the initial count.

(c) Neurones were plated in neurobasal medium supplemented with B27 without antioxidants or in the presence of DNA-damaging agents cytosine arabinoside [AraC] or Etoposide.

(d) Inhibitors of the JNK signalling pathway P600125, ERK signalling pathway PD98059, or phosphatidylinositol 3-kinase signalling pathway Y294002 were added to cultures after the initial count.

No statistically significant differences were revealed between wild-type and $\gamma^{-/-}$ mutant neurones in all cases.


a
100
80
60
40
20
0
% Survival
α+/+/γ+/+
γ-/-
α-/-
α+/+/γ+/+
γ-/-
α-/-
5μg/ml NGF
no NGF
b
100
80
60
40
20
0
% Survival
γ+/+
γ-/-
γ+/+
γ-/-
γ+/+
γ-/-
γ+/+
γ-/-
γ+/+
γ-/-
MG-132
PSI
Cu2+
Zn2+


c
100
80
60
40
20
0
% Survival
γ+/+
γ-/-
γ+/+
γ-/-
γ+/+
γ-/-
γ+/+
γ-/-
no antioxidants
AraC
Etoposide
d
100
80
60
40
20
0
% Survival
γ+/+
γ-/-
γ+/+
γ-/-
γ+/+
γ-/-
γ+/+
γ-/-
SP600125
LY294002
PD98059

3.9 Summary of results

An antibody specific to the C-terminal peptide of mouse γ-synuclein has been successfully purified and has been shown to be effective and specific for use in both western blotting and immunohistochemistry. This antibody was used to perform protein expression studies confirming previous mRNA data. In sensory neurones, axonal and cytoplasmic expression was found to be maintained from E12 into adulthood in both the trigeminal and dorsal root ganglia.

In the SN at E15 both axons and cell bodies are stained however, at E18 this begins to change with the appearance of dotted neuropil staining, which by P2 has replaced the cytoplasmic staining in most cells. In adult animals this replacement is total except in a small number of haphazard immunopositive cell bodies.

Experimental $\gamma^{-/-}$, $\alpha^{-/-}$ synuclein null mutant and $\alpha^{-/-}/\gamma^{-/-}$synuclein double mutant mice were successfully produced using the animals produced by Dr V. Buchman and gifted from Prof A Rosenthal (Genentech). Once on a sufficiently pure genetic background (F6) animals were intercrossed to produce experimental litters. The production of these animals took considerable time so that some experimentation could only be carried out with certain available genotypes.

The loss of α- or γ-synuclein was found to have no effect on the neuronal populations of either the trigeminal or dorsal root ganglia, with no significant differences being found in neuronal number. The population of the cranial motor nuclei was also found to be unchanged in the absence of γ-synuclein.

Furthermore, $\gamma^{-/-}$ neurones have been shown to be equally sensitive to neurotoxic insult as wild-type cells in dissociated primary culture.

In the light of these findings it was decided to focus our efforts on the neuronal populations of the SN. The involvement of synucleins in pathological conditions makes the SN an important target in understanding the role of synucleins in disease. The transgenic models we have developed are excellent tools for gathering valuable information as to the physiological function of this novel protein family.

Chapter 4: RESULTS (II)

4.1 Assaying dopaminergic neurone number

4.1.1 Tyrosine hydroxylase as a dopaminergic marker

To assay the number of dopaminergic (DA) neurones within the *substantia nigra* (SN), we stained histological sections with a specific antibody to tyrosine hydroxylase (TH) (Chapter 2, section 2.7.4). TH is the rate limiting enzyme in the dopamine synthesis pathway, catalysing the hydroxylation of tyrosine to L-3,4-dihydroxyphenylalanine (L-dopa). The importance of TH to murine physiology is underlined by mid-gestational lethality in mice lacking TH (Kobayashi, Morita, Sawada, Mizuguchi, Yamada, Nagatsu, Hata, Watanabe, Fujita and Nagatsu, 1995). Other catecholamines such as adrenalin and noradrenalin have been shown to be severely reduced to <0.5% in TH null foetuses, while tissue levels of dopamine were only reduced to 42% of wild-type levels (Kobayashi et al., 1995). While it is acknowledged that TH is a major enzyme in the dopamine (DA) synthesis pathway, it is not the sole pathway. L-dopa can also be produced from dopaquinone via the oxidation of tyrosine by tyrosinase, part of the melanin synthesis pathway (Riley, 1995). Nevertheless, dopaminergic populations of the SN predominantly utilise the TH synthesis pathway. Therefore this is a good marker for these neurones.

4.1.2 Deficit of dopaminergic neurones in γ-synuclein null mutant ($\gamma^{-/-}$) mice

The number of mesencephalic dopaminergic neurones were assayed at two different points during development (E18, P5) and in adult animals, in both wild-type ($\gamma^{+/+}$) and γ-synuclein null mutant ($\gamma^{-/-}$) mice (Chapter 2, section 2.7.4) These two time points were chosen as they span the period of developmental apoptosis which peaks at P2, as discussed (Chapter 1, section 1.6.2) (Jackson-Lewis et al., 2000). As expression of γ-synuclein begins during development at approximately E12, it is reasonable to hypothesise that the protein has a role to play during normal development. Were γ-synuclein to have a role in apoptosis then a difference between the two genotypes may be observed in neuronal number as development progresses.

To investigate this hypothesis, total numbers of dopaminergic cell bodies were counted i.e. *substantia nigra pars compacta* (SNpc) plus ventral tegmental area (VTA), as embryonic anatomy prevented accurate topographical discrimination between SNpc and VTA. A specific antibody to TH was chosen that clearly labels the perikarya of dopaminergic neurones to enable accurate counting in histological sections.

E18 embryos were dissected from uteri and brains removed, following Schedule 1 protocol. Histological samples were processed and serial coronal sections through the midbrain region were stained with an anti-TH antibody for analysis using fractionator stereology (Chapter 2, section 2.8.2). This method provided an accurate measurement of neuronal number. All experimental data collection was carried out in blind manner to prevent bias.

Analysis of the TH stained sections revealed that no statistically significant differences existed in the total number of dopaminergic neurones present in the primordial SN of $\gamma^{+/+}$ and $\gamma^{-/-}$ animals (Fig. 4.1).

To assess the number of dopaminergic neurones remaining in the SN and VTA of γ-synuclein null mutant mice following the wave of developmental apoptosis at P2, the brains of P5 $\gamma^{+/+}$ and $\gamma^{-/-}$ mice were dissected and processed for immunohistochemistry, as described previously (Chapter 2, section 2.6.3) . In contrast to E18, a small yet significant deficit was observed in the number of TH positive midbrain dopaminergic neurones in $\gamma^{-/-}$ mice at P2 (Fig. 4.1). The mean number of neurones in $\gamma^{-/-}$ (6100 ± 200) being 19% ± 3.2% fewer than the $\gamma^{+/+}$ mice.

To assess whether or not this deficit persisted into adulthood, identical histological methods were applied to the brains of adult $\gamma^{-/-}$ and $\gamma^{+/+}$ mice. A 19% ± 2.4% deficit in the number of dopaminergic neurones of the SN and VTA observed at P5 was still evident in adult $\gamma^{-/-}$ mice (6200 ± 150) as compared to $\gamma^{+/+}$ mice (7600 ± 170; Fig. 4.1).

4.1.3 Effects of the γ-synuclein null mutation on dopaminergic neuronal number in the SN *pars compacta* (SNpc) and ventral tegmental area (VTA)

In adult animals (15-20 weeks old), neurodevelopment is complete and a reliable accurate distinction could be made between the SNpc and VTA (Chapter 2, section 2.7.4). In order to ascertain which of these dopaminergic structures displayed a neuronal deficit, a second investigation was carried out to analyse the SNpc and VTA of adult

mice as separate structures. The boundary between the two structures was drawn applying consistent criteria (Chapter 2, section 2.7.4).

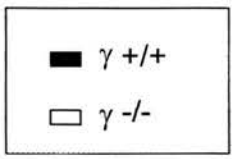
Adult brains were dissected following Schedule 1 protocols and processed for immunohistochemistry as described previously. Sections were again stained with the TH antibody to specifically label dopaminergic neurones and stereology was applied to quantify neuronal number.

Within the SNpc, we observed a very similar statistically significant 14.5% ± 5.2% reduction in the number of TH-positive neurones in the brains of adult $\gamma^{-/-}$ mice (3619 ± 188) in comparison to $\gamma^{+/+}$ (4233 ± 211) mice (Fig. 4.2).

Unlike the SNpc, the VTA of adult $\gamma^{-/-}$ mice (3881 ± 183) displayed no significant change in dopaminergic neuronal number in comparison to $\gamma^{+/+}$ mice (3786 ± 257; Fig. 4.3). These results demonstrate that the developmental deficit described earlier is due to a specific loss of SNpc dopaminergic neurones arising at a point in development between E18 and P5.

Figure 4.1 Number of dopaminergic neurones in midbrain of wild-type and γ-synuclein null mutant mice

This histogram shows the mean ± SEM number of TH-positive neurones in SN + VTA of E18, P5 and adult mice. No difference was found in E18 ($p > 0.5$, Student's *t*-test; $n = 6$ for each genotype) but P5 and adult γ-synuclein null mutant mice ($\gamma^{-/-}$; $n = 8$ and 6, respectively) have significantly less ($^*p < 0.05$, Student's *t*-test) neurones than adult wild-type mice ($\gamma^{+/+}$; $n = 9$). The figure shows that between E18 and P5 a 15-20% deficit arises in the total number of TH-positive cells in the midbrains of γ-synuclein null mutant mice.



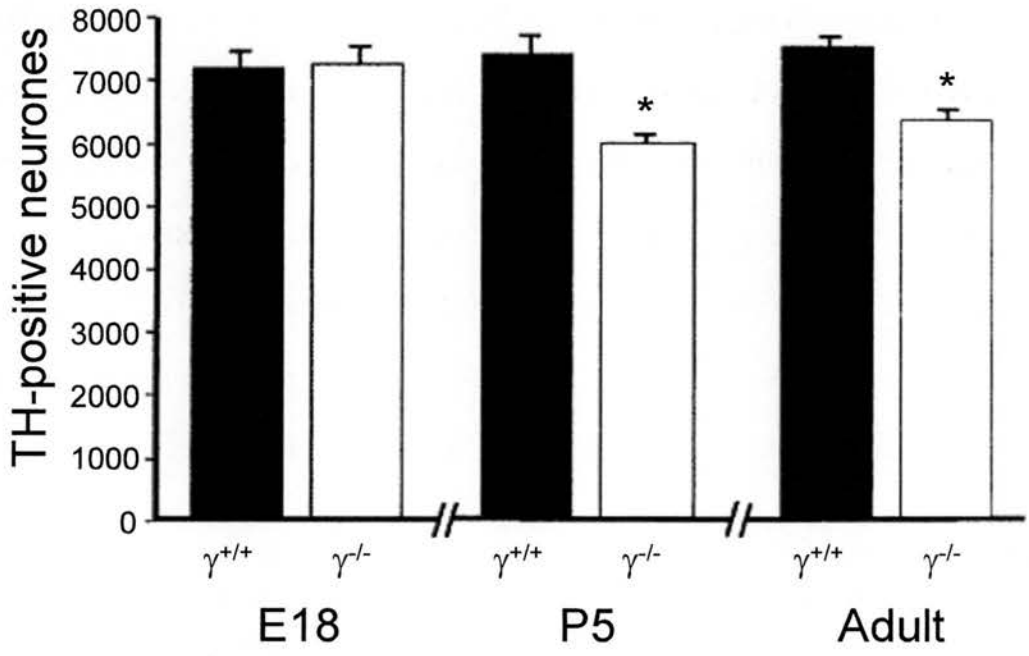
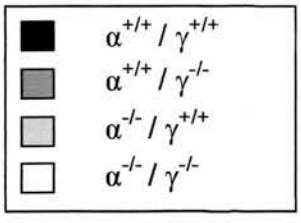

TH-positive neurones
8000
7000
6000
5000
4000
3000
2000
1000
0
*
*
γ+/+
γ-/-
γ+/+
γ-/-
γ+/+
γ-/-
E18
P5
Adult

Figure 4.2 Comparison of number of dopaminergic neurones in the *substantia nigra pars compacta* (SNpc) of γ-, α- or γ/α - synuclein null mutant mice compared to wild-type mice

Histogram shows mean ± SEM number of TH-positive neurones in SNpc of wild-type ($\alpha^{+/+}$ / $\gamma^{+/+}$), γ-synuclein null mutant ($\alpha^{+/+}$ / $\gamma^{-/-}$), α -synuclein null mutant ($\alpha^{-/-}$ / $\gamma^{+/+}$) and double null mutant ($\alpha^{-/-}$ / $\gamma^{-/-}$) mice. Neurones were counted separately in left and right structures of at least seven animals for each genotype. Statistical analysis (Kruskal-Wallis one-way ANOVA) showed a significantly reduced number of neurones in SNpc (*$p < 0.01$) of all three types of mutant mice when compared to wild-type mice. These results clearly show that the developmental deficit in midbrain dopaminergic neurone number exists in the SNpc.



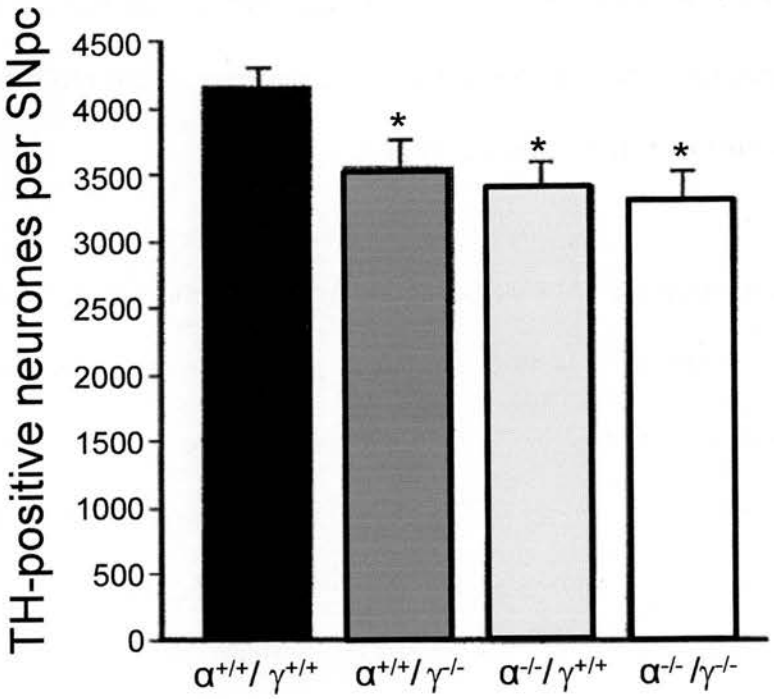
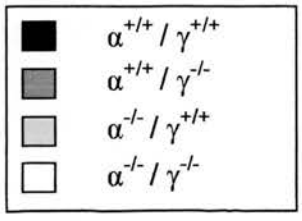
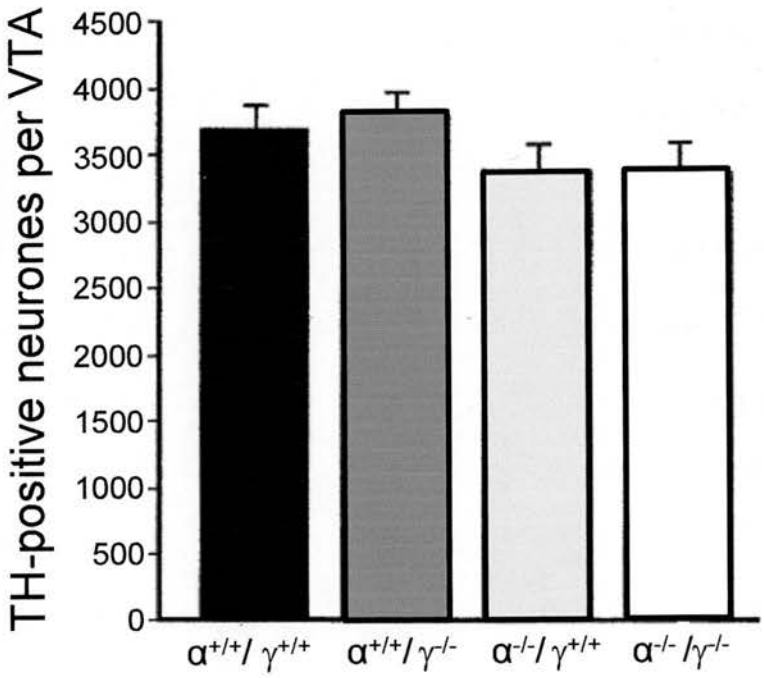

TH-positive neurones per SNpc
4500
4000
3500
3000
2500
2000
1500
1000
500
0
*
*
*
α+/+/ γ+/+
α+/+/ γ-/-
α-/-/ γ+/+
α-/- /γ-/-

Figure 4.3 Comparison of the number of dopaminergic neurones in the ventral tegmental area (VTA) of γ-, α - or γ/α - synuclein null mutant mice compared to wild-type mice

Histogram shows the mean ± SEM number of TH-positive neurones in VTA of wild-type ($\alpha^{+/+}$ / $\gamma^{+/+}$), γ-synuclein null mutant ($\alpha^{+/+}$ / $\gamma^{-/-}$), α-synuclein null mutant ($\alpha^{-/-}$/ $\gamma^{+/+}$) and double null mutant ($\alpha^{-/-}$ / $\gamma^{-/-}$) mice. Neurones were counted separately in left and right structures of at least seven animals for each genotype. Statistical analysis (Kruskal-Wallis one-way ANOVA) showed no difference in VTA ($p > 0.3$) in any mutant mice when compared to wild-type mice. These results clearly show that the developmental deficit in midbrain dopaminergic neurone number is restricted the SNpc and not seen in the VTA.




TH-positive neurones per VTA
4500
4000
3500
3000
2500
2000
1500
1000
500
0
α+/+/ γ+/+
α+/+/ γ-/-
α-/-/ γ+/+
α-/- /γ-/-

4.1.4 Effects of an α-synuclein null mutation on dopaminergic neuronal number in the *substantia nigra pars compacta* (SNpc) and ventral tegmental area (VTA)

In the light of the SNpc specific deficit in the number of DA neurons in $\gamma^{-/-}$ mice an identical study was carried out using α-synuclein null mutant ($\alpha^{-/-}$) mice which became available to us (Chapter 2, section 2.3.2).

Brains were dissected from adult $\alpha^{-/-}$ mice following Schedule 1 methods and processed for immunohistochemistry. Neuronal number was assayed by counting cell bodies stained positive for TH (Chapter 2.7.4).

We observed a statistically significant 17.8% ± 4.4% reduction in the number of SNpc DA neurones in $\alpha^{-/-}$ (3479 ± 186) as compared to wild-type ($\alpha^{+/+}/\gamma^{+/+}$) mice (4233 ± 211; Fig. 4.2). This reduction was almost identical to that seen in $\gamma^{-/-}$ mice. As in $\gamma^{-/-}$ mice, we observed no significant differences in the neuronal number in the VTA of $\alpha^{-/-}$ (3468 ± 183) as compared to $\alpha^{+/+}/\gamma^{+/+}$ mice (3786 ± 257; Fig. 4.3).

4.1.5 Effects of a double α-/γ-synuclein null mutation on dopaminergic neuronal number in the SNpc and ventral tegmental area (VTA)

A statistically significant difference in the number of dopaminergic SNpc neurones was observed in both $\alpha^{-/-}$ and $\gamma^{-/-}$ mice. Following the generation of double α-/γ-synuclein double null mutant animals ($\alpha^{-/-}/\gamma^{-/-}$) (Chapter 2, section 2.3.3), an identical investigation was carried out to elucidate whether or not any cumulative effects of the mutations would occur, producing a larger deficit in the SNpc or perhaps precipitating one in the VTA, where previously no differences were observed.

Identical histological methods were applied to brains of $\alpha^{-/-}/\gamma^{-/-}$ mice, dissected according to Schedule 1 protocols. A statistically significant 20.1% ± 5.3% reduction was observed in the dopaminergic neurones of the SNpc of $\alpha^{-/-}/\gamma^{-/-}$ mice (3382 ± 179) as compared to $\alpha^{+/+}/\gamma^{+/+}$ mice (4233 ± 211), such that no cumulative effects from the double null mutation were observed (Fig. 4.2). The same pattern as previously seen was observed in the VTA i.e. no significant differences existed in DA neuronal number between $\alpha^{-/-}/\gamma^{-/-}$ (3460 ± 204) and $\alpha^{+/+}/\gamma^{+/+}$ mice (3786 ± 257; Fig. 4.3).

As these data were collected in three independent studies, it is worth noting that each genotype was compared to wild-type ($\alpha^{+/+}/\gamma^{+/+}$) animals individually. As no differences were observed in the numbers of neurones in $\alpha^{+/+}/\gamma^{+/+}$ animals, the data could be combined.

4.2 Motor performance assessment in synuclein null mutant mice

The role of the SN in motor control is now well characterised (Chapter 1, section 1.6.1). Therefore, it was logical to assess the motor performance of synuclein null mutant mice to investigate whether or not the neuronal deficit observed could translate into a deleterious phenotype in the form of a deficit in motor performance.

4.2.1 Motor performance of synuclein null mutant mice at constant speed

Groups of adult male $\alpha^{+/+} / \gamma^{+/+}$, $\alpha^{+/+} / \gamma^{-/-}$, $\alpha^{-/-} / \gamma^{+/+}$ and $\alpha^{-/-} / \gamma^{-/-}$ animals were assessed for motor performance using an Ugo Basile rotarod. Animals were given three two minute long training periods on the equipment before experimental data were recorded. Animals were placed onto the rotating rod in individual compartments and time measured until the animals fell from the rod as described above (Chapter 2, section 2.10.1).

At a speed of 24 revolutions per minute (rpm), wild-type performance was taken as the mean 100% performance value. Single null mutant $\alpha^{+/+} / \gamma^{-/-}$ and $\alpha^{-/-} / \gamma^{+/+}$ mice showed no observable significant differences in motor performance compared to wild-type animals (Fig. 4.4). The double null mutant $\alpha^{-/-} / \gamma^{-/-}$ mouse group, while perhaps the most likely group to show a retarded motor performance, also showed no observable differences in performance when compared to $\alpha^{+/+} / \gamma^{+/+}$ or single null mutant $\alpha^{+/+} / \gamma^{-/-}$ and $\alpha^{-/-} / \gamma^{+/+}$ mice (Fig. 4.4).

4.2.2 Motor performance of synuclein null mutant mice in an accelerating trial

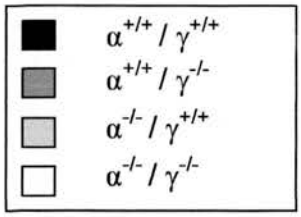
The animals described above were again placed on the rod in individual compartments. However in this accelerating trial the rod was initially stationary but accelerated steadily to 40 rpm over five minutes and the clock was stopped when the animal fell from the rod.

Once again, results followed the pattern of the previous experiment, with no significant differences in performance being observed between $\alpha^{+/+} / \gamma^{+/+}$ mice and the synuclein null mutant $\alpha^{+/+} / \gamma^{-/-}$, $\alpha^{-/-} / \gamma^{+/+}$ and $\alpha^{-/-} / \gamma^{-/-}$ mice (Fig. 4.5).

We concluded from these investigations that the developmental neuronal deficit within the SNpc of synuclein deficient mice does not translate into impaired motor function.

Figure 4.4 Performance of wild-type and synuclein null mutant mice in constant speed rotorod tests

Histogram shows mean ± SEM of time intervals from the test start to animal fall from the rotating rod. Results of 3 minute test with constant 24 rpm. Statistical analysis (Kruskal-Wallis one-way ANOVA) showed no significant difference in the performance of wild-type and mutant mice ($p > 0.4$).



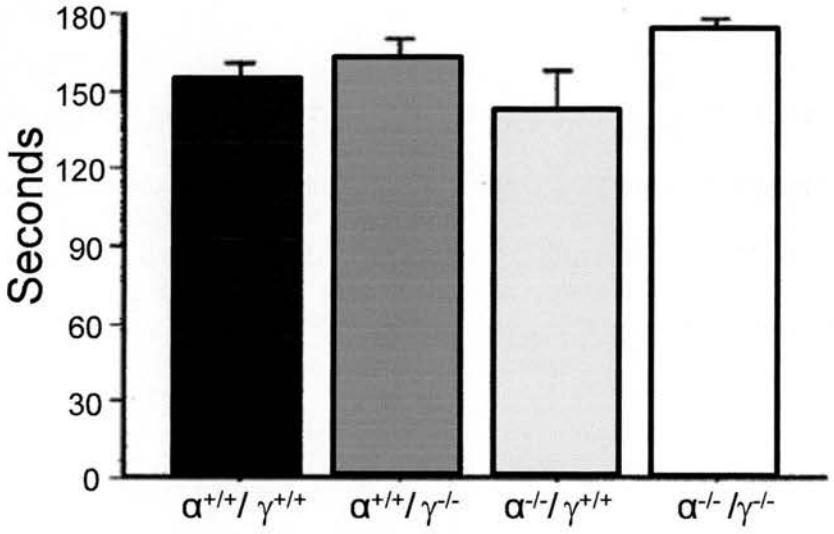
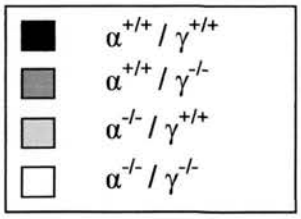
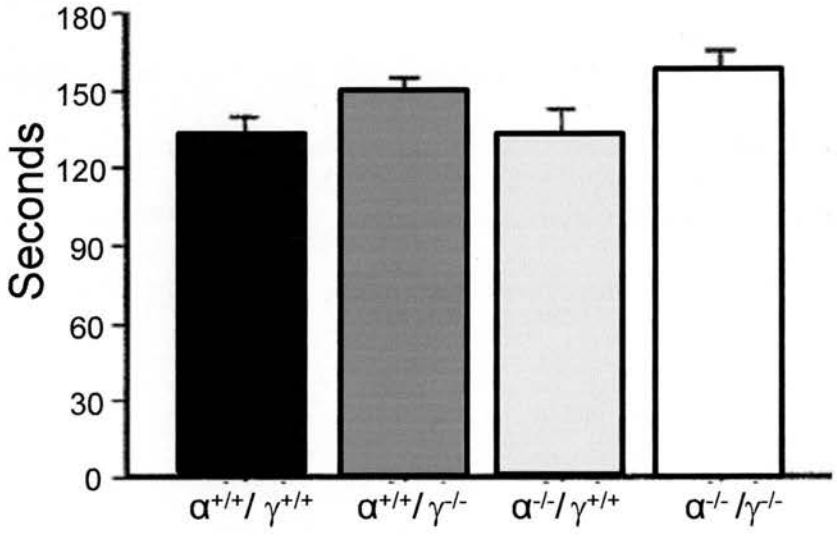

Seconds
180
150
120
90
60
30
0
α+/+/ γ+/+
α+/+/ γ-/-
α-/-/ γ+/+
α-/- /γ-/-

Figure 4.5 Performance of wild-type and synuclein null mutant mice in accelerating rotarod tests

Histogram shows mean ± SEM of time intervals from the test start to animal falling from the rotating rod. Results of 5 min accelerating rotation test are shown. Statistical analysis (Kruskal-Wallis one-way ANOVA) showed no significant difference in performance of wild-type and mutant mice in both tests ($p > 0.4$).




180
150
120
90
60
30
0
Seconds
α+/+/ γ+/+
α+/+/γ-/-
α-/-/γ+/+
α-/- /γ-/-

4.3 Assaying levels of dopamine and its metabolites in the striatum of synuclein null mutant mice

4.3.1 Assaying levels of dopamine and its metabolites via HPLC analysis

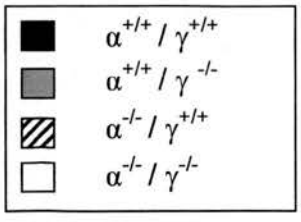
To assess the effect of the synuclein null mutation and the reduction in dopaminergic neuronal number on the levels of dopamine and its metabolites, the striatum was dissected from nine month old male mice. High performance liquid chromatography (HPLC) analysis was used to measure dopamine (DA), 5-hydroxyindolacetic acid (5-HIAA), 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) levels in the extract of each of individual striatum. The HPLC analysis itself was carried out by Paul Jones and John Sharkey of the Fujisawa Institute of Neuroscience, Edinburgh and as in other experiments, performed blindly and is described above (Chapter 2, section 2.11).

Brains of nine month old male $\alpha^{+/+}/\gamma^{+/+}$, $\alpha^{+/+}/\gamma^{-/-}$, $\alpha^{-/-}/\gamma^{+/+}$ and $\alpha^{-/-}/\gamma^{-/-}$ mice were dissected and snap frozen for storage prior to analysis on an HPLC column. Samples were later defrosted and homogenised before extracts were injected onto the column.

Levels of all four neuro-chemicals studied, DA, HIAA, DOPAC and HVA were analysed and $\alpha^{+/+}$ / $\gamma^{+/+}$ levels normalised as 100% for comparison against $\alpha^{+/+}$ / $\gamma^{-/-}$, $\alpha^{-/-}$ / $\gamma^{+/+}$ and $\alpha^{-/-}$ / $\gamma^{-/-}$ samples. The synuclein null mutant samples showed no significant reductions in the striatal level of either dopamine or its metabolites HIAA, DOPAC or HVA (Fig. 4.6 and Fig. 4.7). These results indicated that the reduction in neuronal number caused by these null mutations was not significant enough to precipitate a drop in levels of striatal dopamine.

Figure 4.6 Dopamine and its metabolite 5-hydroxyindolacetic acid levels in striatum of wild-type and synuclein null mutant mice

Striatal concentrations (ng/mg protein) of (a) dopamine (DA) and (b) 5-hydroxyindolacetic acid (5-HIAA) in mutant animals were normalised to corresponding mean values for wild-type animals (100%) in each experiment. Mean ± SEM for nine animals per genotype from two separate experiments are shown. Statistical analysis showed no significant difference between wild-type and mutant mice for either of the neurochemicals ($p > 0.1$, one-way ANOVA with posthoc Newman-Keuls test).



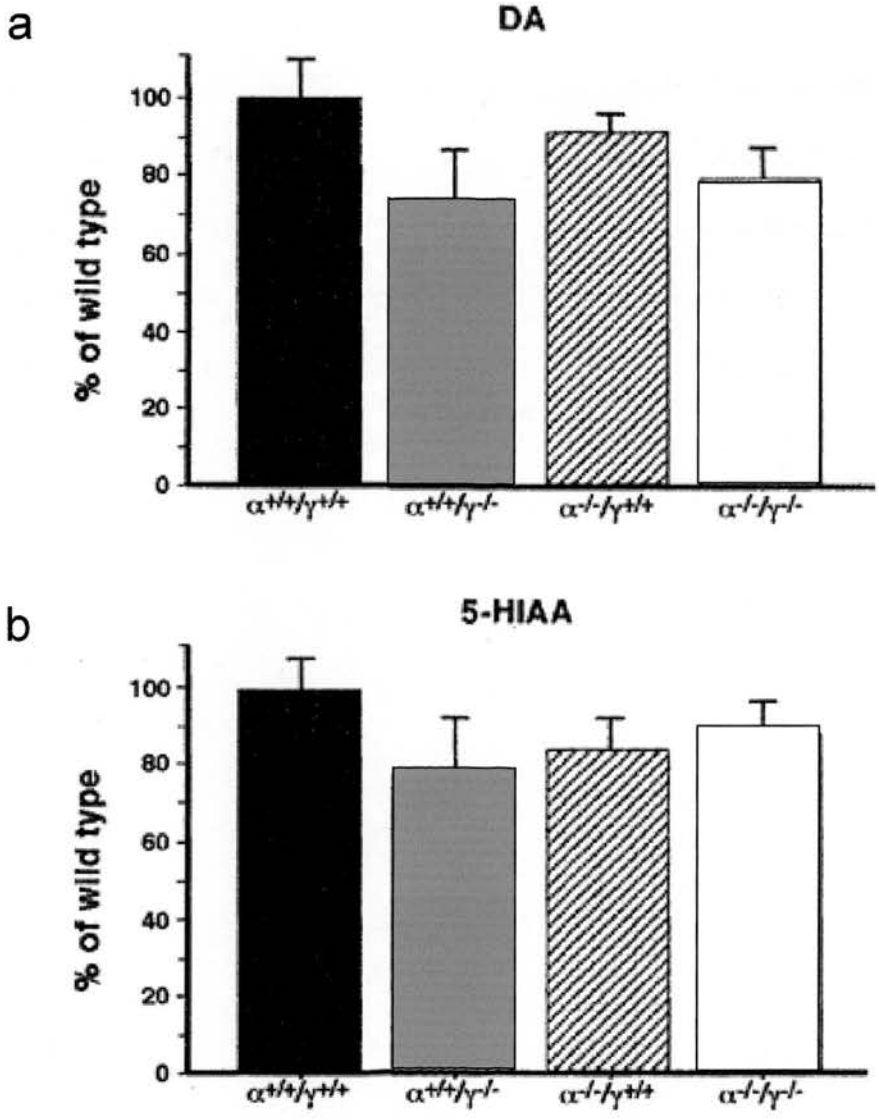
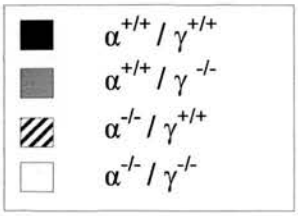
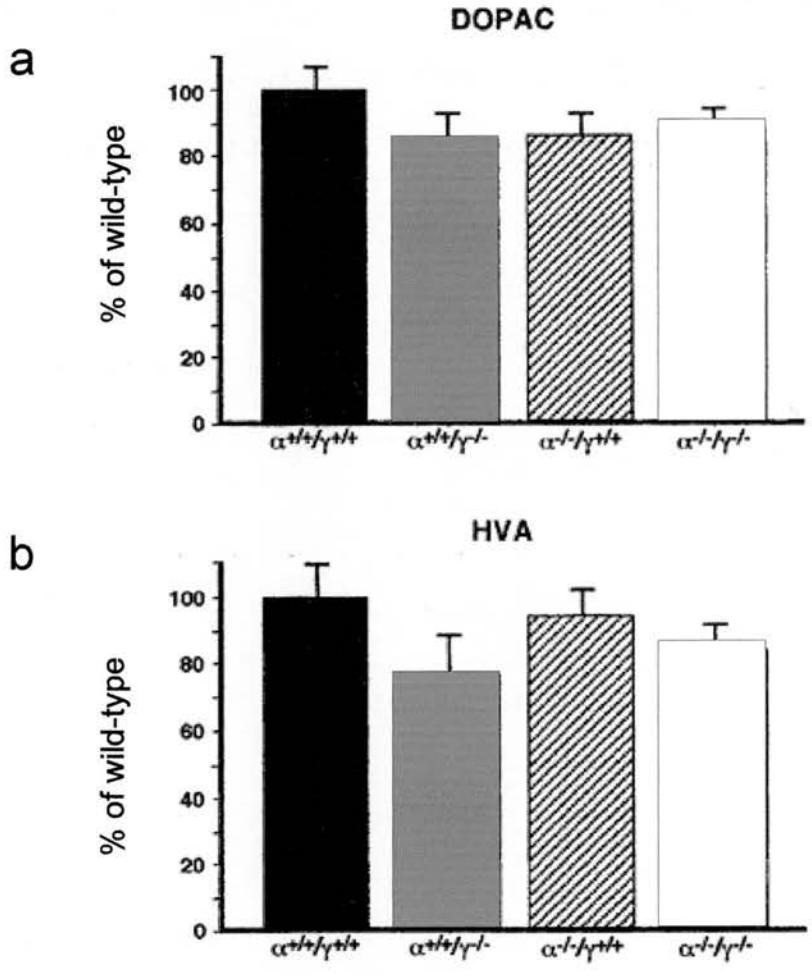

a
DA
% of wild type
100
80
60
40
20
0
α+/+/γ+/+
α+/+/γ-/-
α-/-/γ+/+
α-/-/γ-/-
b
5-HIAA
% of wild type
100
80
60
40
20
0
α+/+/γ+/+
α+/+/γ-/-
α-/-/γ+/+
α-/-/γ-/-

Figure 4.7 Levels of dopamine metabolites 3,4-dihydroxyphenylacetic acid and homovanillic acid in striatum of wild-type and synuclein null mutant mice

Striatal concentrations (ng/mg protein) of (a) 3,4-dihydroxyphenylacetic acid (DOPAC) and (b) 5-hydroxyindolacetic acid (HVA) in mutant animals were normalised to corresponding mean values for wild-type animals (100%) in each experiment. Mean ± SEM for nine animals per genotype from two separate experiments are shown. Statistical analysis showed no significant difference between wild-type and mutant mice for either of the neurochemicals ($p > 0.1$, one-way ANOVA with posthoc Newman-Keuls test).




a
DOPAC
% of wild-type
100
80
60
40
20
0
α+/+/γ+/+
α+/+/γ-/-
α-/-/γ+/+
α-/-/γ-/-
b
HVA
% of wild-type
100
80
60
40
20
0
α+/+/γ+/+
α+/+/γ-/-
α-/-/γ+/+
α-/-/γ-/-

4.4 Changes in sensitivity to neurotoxic insult of dopaminergic neurones in synuclein null mutant mice

4.4.1 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)

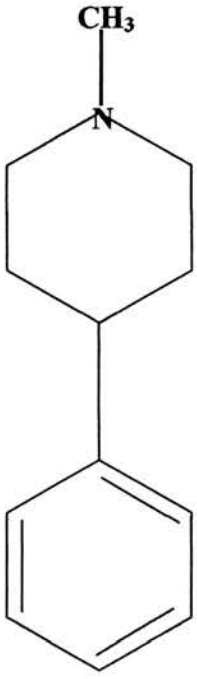
The biological effects of the potent neurotoxin MPTP (1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine) were first noted in July of 1982 in northern California (Langston, Ballard, Tetrud and Irwin, 1983). MPTP had been ad-mixed with MPPP (1-Methyl-4-phenyl-4-propionoxypiperidine) and sold as synthetic heroin in this limited region of the United States. Initially, one female and three male patients were admitted to hospital with severe Parkinson-like symptoms, having taken between 5-20g of the drug intravenously over a period of days. All patients became symptomatic within one week of taking the drug. They began with visual hallucinations, jerking and generalised muscle stiffness and dyskinesia which continued to develop despite the discontinued use of the drug. After 6 weeks, each of the patients was found to be all but immobile with generalised increase in muscle tone, fixed stare and constant drooling. All patients were found positive in the Glabellar tap test and displayed the classical pill rolling tremor, in addition to general bradykinesia. All individuals did however respond to Levodopa and Carbidopa therapy although none displayed any sign of remission (Langston et al., 1983). Following these cases, it became apparent that this compound could prove a useful tool in the development of an in *vivo* model in primates and rodents to investigate Parkinson's disease (PD) (Kolata, 1983). It was found that MPTP is initially metabolised by type B monoamine oxidase (EC1.4.3.4, MAO-B), predominantly glial in origin, into the intermediate 1-methyl-4-phenylpyridinium ($MPDP^+$) (Ramsay and Singer, 1986). In

a further oxidation, the final four-electron oxidation product, the actual toxic molecule, 1-methyl-4-phenylpyridinium (MPP^+), is produced. The toxic intracellular accumulation of MPP^+ occurs as it is a high affinity substrate for the dopamine transporter (DAT) due to the structural similarities between itself and dopamine (Fig. 4.7) (Javitch, D'Amato, Strittmatter and Snyder, 1985).

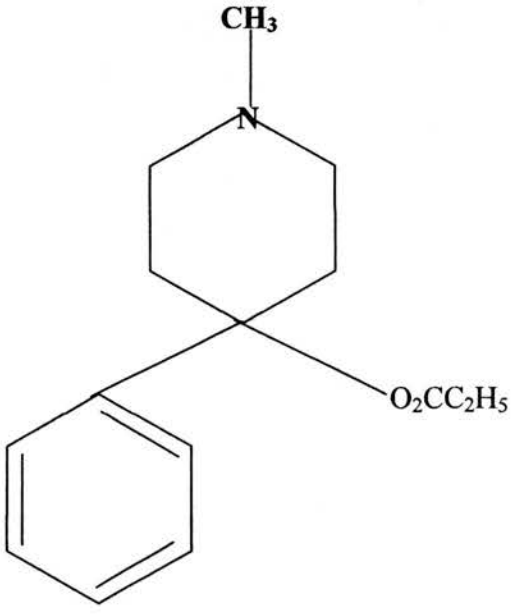
Figure 4.8 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)

Line diagrams showing the chemical structure of 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and its toxic metabolite 1-methyl-4-phenylpyridinium (MPP^+) for comparison with that of the neurotransmitter dopamine.

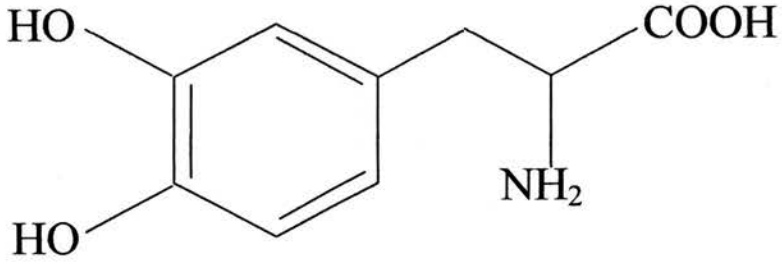
MPTP



MPP$^+$



Dopamine



4.4.2 Dopaminergic neurones of single and double synuclein deficient mice are resistant to MPTP toxicity

To address the question of whether the absence of synucleins might alter the sensitivity of dopaminergic neurones to specific neurotoxic insults, we treated $\alpha^{+/+}/\gamma^{+/+}$, $\alpha^{-/-}$, $\gamma^{-/-}$ and $\alpha^{-/-}/\gamma^{-/-}$ null mutant mice with MPTP, a neurotoxic drug that predominantly affects dopaminergic neurones. A protocol of MPTP administration (Chapter 2, section 2.9) that results in the apoptotic death of dopaminergic neurones was chosen (Tatton and Kish, 1997). This protocol allowed for the identification of animals more sensitive as well as less sensitive to the neurotoxic effect of the drug because it causes only a moderate reduction, approximately 40%, in the number of dopaminergic neurones of the SNpc of wild-type mice. The deficit precipitated is not accompanied by reductions in the level of striatal dopamine.

The experimental cohort of mice that were to be given the course of i.p. MPTP injections were separated from the general population 1 week prior to the first injection of the drug, for health and safety reasons and to ensure control animals were not contaminated. Five daily 30mg/kg doses of MPTP, injections at 10mg/ml were administered to the experimental group and sterile PBS given to the control (vehicle) group. Twenty one days after the last injection, brains were dissected following Schedule 1 procedures, and were processed for histology as described above. An antibody to tyrosine hydroxylase (TH) was used to stain the dopaminergic neurones of the SNpc (Chapter 2, section 2.7.4).

Wild-type animals demonstrated the expected reduction in TH-positive neuronal number following the MPTP injection regimen. 64.7 ± 11.1% of neurones remained in the MPTP-treated group; 100% was the mean of neurone numbers in a vehicle treated group of wild-type animals (Fig. 4.9). All other data are expressed as a percentage survival as compared with the vehicle injected equivalent genotype and not compared to wild-type. Interestingly, no statistically significant reductions were observed within the synuclein null mutant cohorts. $\alpha^{-/-}$ animals retained 88.1 ± 4.7% of neurones in the MPTP-treated group as compared with vehicle injected animals (Fig. 4.9). $\gamma^{-/-}$ mice retained 95.3 ± 7.4% of neurones in the MPTP-treated group as compared to vehicle injected $\gamma^{-/-}$ mice (Fig. 4.9). $\alpha^{-/-}/\gamma^{-/-}$ animals again showed no cumulative effects of the mutation, retaining 85.9 ± 6.6% of neurones following MPTP treatment (Fig. 4.9).

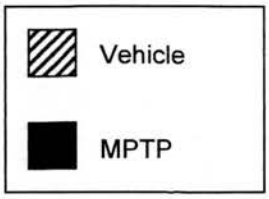
4.4.3 Effect of chronic MPTP treatment on the motor performance of wild-type and synuclein null mutant mice

The same experimental cohort of animals used in the experiment described above were tested two weeks following administration of MPTP or saline vehicle in an accelerating rotorod trial as described previously (Chapter 2, section 2.10.1).

No significant differences in motor performance were observed in the $\alpha^{+/+}/\gamma^{+/+}$ group in either MPTP or saline injected groups (Fig. 4.10). Furthermore, none of the $\alpha^{-/-}$, $\gamma^{-/-}$ or the $\alpha^{-/-}/\gamma^{-/-}$ groups revealed any differences in motor performance between the MPTP treated animals in comparison with their saline injected control group (Fig. 4.10).

Figure 4.9 Effect of chronic MPTP treatment on wild-type and synuclein null mutant dopaminergic neurones of the SNpc

The number of dopaminergic neurones in SNpc of wild-type and synuclein null mutant treated with MPTP or vehicle. Neurones were counted separately in left and right SNpc of at least six animals for each experimental group. Statistical analysis (*p<0.2, Kruskal-Wallis one-way ANOVA and Student's *t*-test separately for each genotype) showed a significant reduction in the number of neurones after MPTP treatment only for wild-type animals.



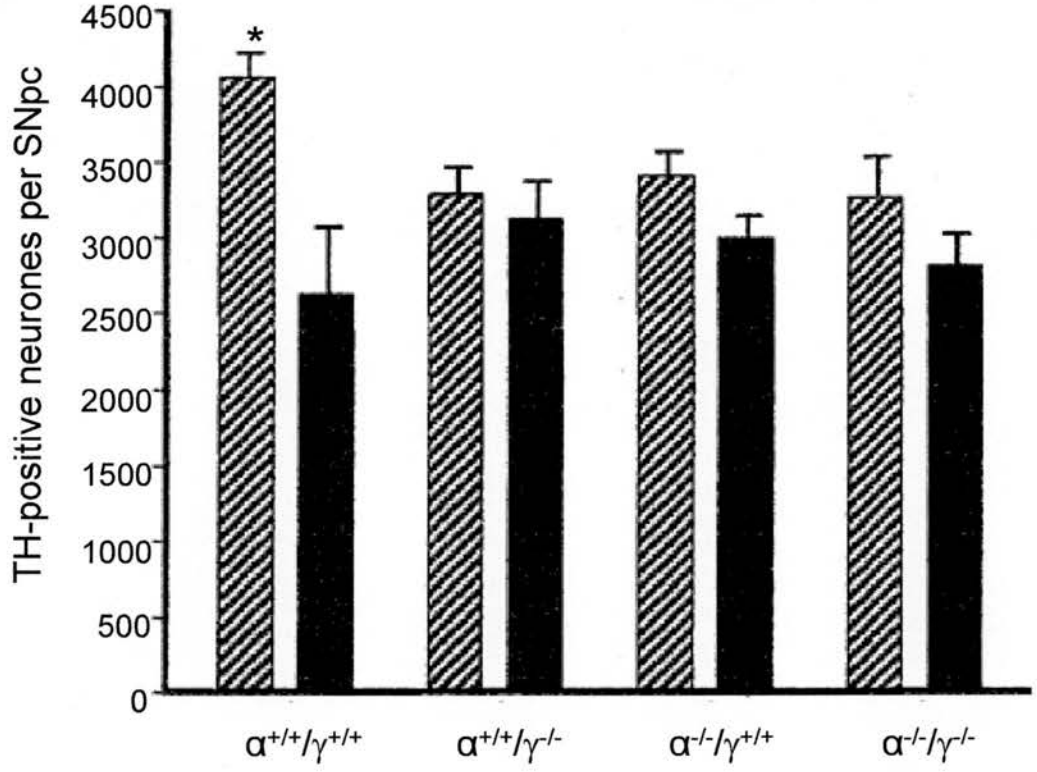
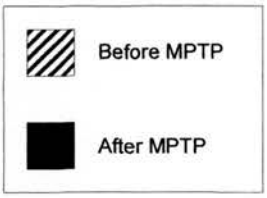
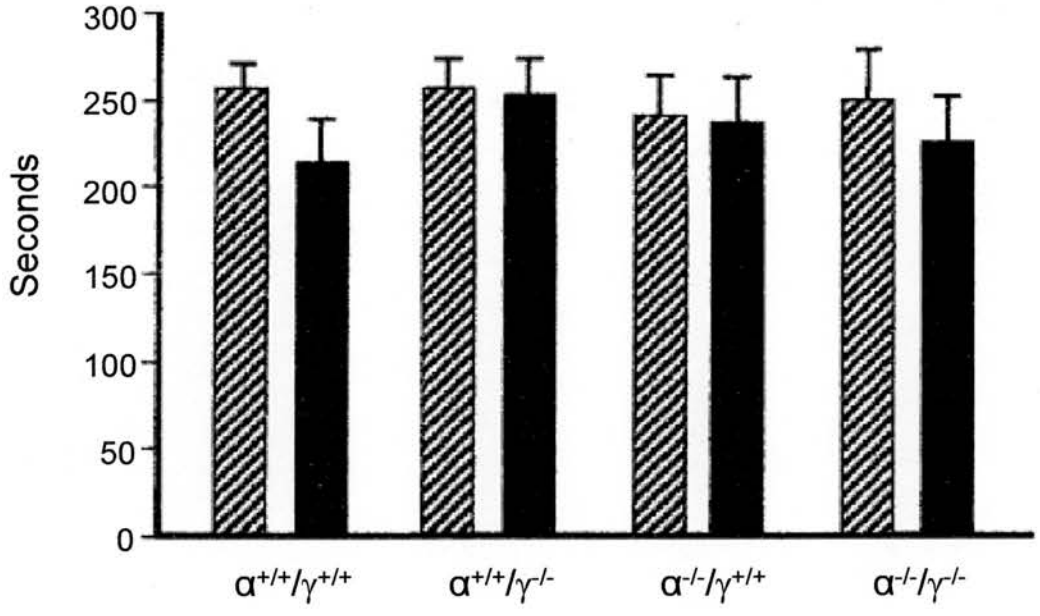

TH-positive neurones per SNpc
4500
4000
3500
3000
2500
2000
1500
1000
500
0
*
α+/+/γ+/+
α+/+/γ-/-
α-/-/γ+/+
α-/-/γ-/-

Figure 4.10 Effect of chronic MPTP treatment on the motor performance of wild-type and synuclein null mutant mice

Performance of wild-type ($\alpha^{+/+}/\gamma^{+/+}$), γ-synuclein null mutant ($\alpha^{+/+}/\gamma^{-/-}$), α-synuclein null mutant ($\alpha^{-/-}/\gamma^{+/+}$) and double null mutant ($\alpha^{-/-}/\gamma^{-/-}$) mice in 5 min accelerating rotarod test before and 2 weeks after MPTP treatment. No significant differences in motor performance arose following MPTP administration.




Seconds
300
250
200
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100
50
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α+/+/γ+/+
α+/+/γ-/-
α-/-/γ+/+
α-/-/γ-/-

4.5 Synuclein expression in the midbrain of null mutant mice

4.5.1 Assaying synuclein protein level

The relative expression level of the remaining un-mutated synuclein proteins were investigated in the three synuclein null mutant colonies. This also allowed us to verify the total absence of α- and γ-synuclein protein from our double null mutant mice. Midbrain tissues were dissected from adult male $\gamma^{-/-}$, $\alpha^{-/-}$, $\alpha^{-/-}/\gamma^{-/-}$ and $\alpha^{+/+}/\gamma^{+/+}$ mice and homogenised in Laemmli lysis buffer as described above (Chapter 2, section 2.5.1). 10μg of total midbrain protein lysate from each genotype were loaded into separate lanes on three agarose gels prepared and run as described previously (Chapter 2, section 2.5.2). The separated proteins were transferred onto 3 nylon membranes which were then probed with antibodies to α-, β- and γ-synuclein. The filter probed with an anti-β-synuclein antibody was stripped and re-probed with an anti-α-tubulin antibody to ensure equivalent amounts of total protein were loaded.

The $\gamma^{-/-}$ tissue showed a complete absence of γ-synuclein protein as expected and the level of α-synuclein expression was equivalent to the $\alpha^{+/+}/\gamma^{+/+}$ control sample (Fig. 4.11). However, the level of β-synuclein protein expression appeared to be elevated in comparison with the $\alpha^{+/+}/\gamma^{+/+}$ control sample (Fig. 4.11).

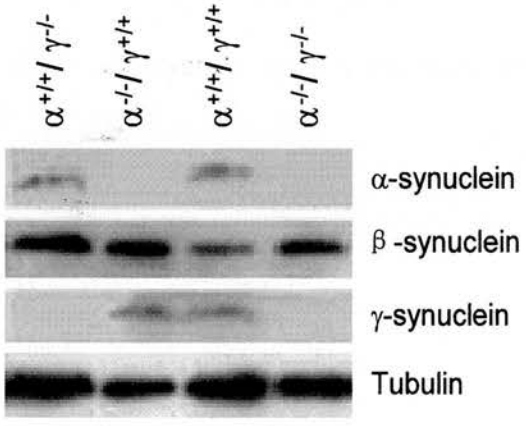
Similarly, the $\alpha^{-/-}$ tissue showed a complete absence of α-synuclein protein as expected and the level of γ-synuclein expression was equivalent to the $\alpha^{+/+}/\gamma^{+/+}$ control sample (Fig. 4.11). Again, an increase in β-synuclein expression was detected (Fig. 4.11).

The $\alpha^{-/-}/\gamma^{-/-}$ tissue confirmed the absence of both proteins from these animals as expected and again an increase in β-synuclein protein expression was observed (Fig. 4.11). In

both the single and double null mutant animals, there was an equivalent increase in β-synuclein expression.

Figure 4.11 Western blot showing synuclein protein expression in midbrain of wild-type and synuclein null mutant mice

Each of the three identical western blots with 10μg of total midbrain protein lysates per lane were probed with antibodies against α-synuclein, β-synuclein and γ-synuclein. The filter probed with β-synuclein was stripped and re-probed with anti-α-tubulin as a control (lower panel). Midbrains of adult male $\gamma^{-/-}$, $\alpha^{-/-}$, $\alpha^{-/-}/\gamma^{-/-}$ and $\alpha^{+/+}/\gamma^{+/+}$ mice are compared (four combined midbrains for each genotype).


α+/+ / γ-/-
α-/- / γ+/+
α+/+ / γ+/+
α-/- γ-/-
α-synuclein
β -synuclein
γ-synuclein
Tubulin

4.6 Summary of results

A 15-20% developmental deficit in the total number of dopaminergic neurones in the SNpc and VTA was detected at P5 in $\gamma^{-/-}$ mice. We have shown this deficit must arise between E18 and P5 as at E18 there were no significant differences. The reduction was also seen in adult animals.

To identify the source of this reduction, a study was carried out in adult $\gamma^{-/-}$, $\alpha^{-/-}$ and $\alpha^{-/-}$ / $\gamma^{-/-}$ mice animals to identify which structures were responsible for the reduction as a distinction was now possible between the SNpc and the VTA. The 15-20% reduction in neuronal number was restricted to the SNpc of all mutant mice studied, with no effects observed in the VTA of any group.

In light of the neuronal deficit displayed by the null mutant animals in the SNpc, an area associated with motor behaviour, we tested these animals on a rotorod looking for any signs of impaired motor function. Two tests were carried out, one at a constant speed of 24 rpm and another accelerating from 0 to 40 rpm over the 5 minute duration of the test. No statistically significant differences were observed between any mutant groups and their wild-type littermates.

The levels of striatal dopamine and its metabolites 5-hydroxyindolacetic acid, 3,4-dihydroxyphenylacetic acid and homovanillic acid were assayed using HPLC analysis to detect whether or not the reduction in dopaminergic neurones seen in the SNpc translated into a reduction in striatal dopamine levels. However, no statistically significant differences were observed between the three null mutant groups and their wild-type littermates.

MPTP is a DA specific neurotoxin which has become a widely used to create models of Parkinson's disease in rodents and primates. So, we tested the sensitivity of $\alpha^{-/-}$, $\gamma^{-/-}$ and $\alpha^{-/-}/\gamma^{-/-}$ mice to the potent neurotoxin MPTP. $\alpha^{+/+}/\gamma^{+/+}$ animals showed the typical 40% reduction seen with our chosen dose regimen as compared to vehicle injected controls. All three mutant groups however showed complete insensitivity to the drug as no changes in neurone number were observed in comparison with a vehicle injected control group of the same genotype.

These same animals showed equivalent performance in an accelerating rotorod trial with no differences being observed between genotypes or MPTP and control injected controls.

Western blotting analysis of midbrain tissue confirmed the absence of α- and γ-synuclein protein from our $\alpha^{-/-}/\gamma^{-/-}$ mice. In addition an increase in β-synuclein expression was detected in all synuclein mull mutant strains in comparison with $\alpha^{+/+}/\gamma^{+/+}$ animals.

The synuclein null mutants generated in the laboratory have allowed us to generate a wealth of data, which will doubtless contribute to the elucidation of the physiological function of this novel protein family.

Chapter 5: Discussion

Compelling evidence implicates the synuclein family of proteins in various neurodegenerative conditions such as Parkinson's disease and dementia with Lewy bodies. The focus of synuclein research to date has largely been directed towards α-synuclein, which has been shown to be neurotoxic and involved in proteosomal protein degradation.

The findings presented herein are an attempt to redress this imbalance; to this end we focused our attention initially on γ-synuclein and its sub-cellular localisation within certain neuronal populations and the effects of a null mutation on murine physiology and development. To begin these investigations an antibody specific to mouse γ-synuclein was purified to assess the distribution of γ-synuclein in structures throughout the nervous system.

5.1 Affinity purified anti γ-synuclein antibody

An antibody to the C-terminal peptide of mouse-γ-synuclein was successfully raised in rabbit and the antiserum generated was purified via a GST-fusion protein column. It was crucial to create our own unique highly specific antibody as no commercial alternative was available and having such a tool was essential to our investigations. The antibody that I have purified has been shown to be effective for use in Western blotting at a dilution of 1:200 for the detection of γ-synuclein protein in mouse tissues. We have clearly demonstrated using γ-synuclein null mutant ($\gamma^{-/-}$) mouse tissue that the antibody was specific for the detection of γ-synuclein only, showing no cross-reactivity with either α- or β- synuclein. In addition, the antibody was used

for the specific detection of γ-synuclein protein in immunohistochemistry and immunofluorescence studies.

This antibody was used in histological studies to confirm that protein distribution was consistent with previous data localising γ-synuclein mRNA in several structures throughout the developing nervous system (Buchman et al., 1998; Lavedan et al., 1998a; Abeliovich et al., 2000). This revealed the subtleties of intracellular compartmentalisation of γ-synuclein in various neuronal populations at distinct points during development. The importance of the specificity of the antibody cannot be overstated as previous protein localisation studies, such as those by Li and colleagues used an antibody raised against complete recombinant human γ-synuclein, applying them to rat brain (Li et al., 2002). This is undesirable as γ-synuclein exhibits the lowest degree of interspecies homology of the three synuclein proteins (Lavedan et al., 1998a). As the immunogenic peptide was full length human γ-synuclein, the antibody produced may also react to regions of the N-terminal which is highly conserved between all synucleins; hence cross reactivity becomes a concern. The antibody that we have purified is unique and specific for a region within the C-terminal of mouse γ-synuclein. Using this antibody, we were able to define the localisation pattern of γ-synuclein in specific neuronal populations for the first time.

5.2 γ-synuclein localisation

5.2.1 Localisation of γ-synuclein in sensory neurones

High levels of γ-synuclein were detected via immunohistochemistry in the sensory populations of the dorsal root ganglia (DRG) which arise from neural crest tissue and trigeminal ganglia (TG) which arise from the placode. The conclusions drawn from these investigations were from findings of consistent staining patterns observed in at least 4 animals; these criteria were maintained for all expression studies.

At E12, this expression was present in both the neuronal cell bodies and axons of all neurones within both these structures. This expression was maintained throughout embryonic development into the postnatal period at the same high level. At this time, neuronal populations have matched the requirement of their target field for innervation and following a period of natural cell death, maturation can proceed. Any changes in compartmentalisation related to potential changes in function are likely to have occurred by this time, around P2. However, we observed no such changes. We may have observed changes in localisation if the role of γ-synuclein was changing during development; however this did not appear to be the case in either the TG or DRG.

Damage to the sensory neurones in the DRG can cause abnormal pain processing. As γ-synuclein expression is abundant in DRG, it was of interest to examine whether γ-synuclein was involved in a hypersensitive state following damage to these primary afferent neurones. Thus, in a parallel study, $\gamma^{-/-}$ mice underwent a model of peripheral nerve injury to induce a neuropathic pain state. This manifests as a behavioural sensitisation that is recorded as an increase hind paw withdrawal reflex response to sensory stimuli. Prior to injury, $\gamma^{-/-}$ mice had the same response as wild-type

littermates indicating that γ-synuclein does not play a role in normal nociceptive processing. At the time of maximal behavioural sensitisation resulting from nerve injury, $\gamma^{-/-}$ mice had the same expected sensitised responses as their wild-type littermates in tests for both a noxious thermal stimulus and an innocuous mechanical stimulus (Ninkina et al., 2003). This indicates that γ-synuclein does not play a role in the abnormal pain processing that occurs following nerve injury, thus neuronal function was not significantly affected.

Several null mutant animal models have been shown to evoke changes in neuropathic sensitivity; RI-β the regulatory subunit of PKA for example (Malmberg, Brandon, Idzerda, Liu, McKnight and Basbaum, 1997). The high level of γ-synuclein expression in the DRG made this model an excellent test of *in-vivo* neuronal function. Initial *in-situ* hybridisation studies revealing embryonic expression of mRNA in the DRG appear to show a decreasing gradient of expression moving caudally down the spinal cord (Buchman et al., 1998). Logically then the DRG L4-6 innervated by the sciatic nerve may not be the most sensitive region to target, however the nature of the model places restrictions on testing regions of greater expression.

Significantly, a different trend in γ-synuclein localisation was observed in a complementary study carried out in our laboratory by Dr. O. Schmidt, into γ-synuclein expression in the cranial motor nuclei arising from the medial and lateral parts of the basal lamina. In the hypoglossal, facial, trochlear, oculomotor and trigeminal motor nuclei at E12 the pattern of γ-synuclein expression mirrored exactly that seen in sensory neurones, i.e. intense cell body and axonal staining (Ninkina et

al., 2003). However, at E18 this pattern began to change with the appearance of dotted neuropil staining at the cell periphery, which replaced the cytoplasmic staining in most cells by P2. In adult animals, this replacement was total except in the facial nuclei, which retained several haphazard immunopositive cell bodies. In the other structures, only axons and nerve terminals displayed staining (Ninkina et al., 2003).

These findings are in contrast to those of Li and colleagues who report the presence of γ-synuclein-positive cell bodies in the motor nuclei of adult rats (Li et al., 2002). It is unlikely that these differing observations are a result of interspecies differences between rodents and, given the high degree of homology between proteins, it is also unlikely they are performing different functions in rats and mice. As mentioned previously, the antibody used by Li et al (2002). was raised against recombinant human γ-synuclein and tested for specificity against the same human protein. Significantly, this antibody was not validated for rat γ-synuclein or on null mutant negative control tissue.

It is unknown why a sub-population of cells within the facial nuclei display the same expression pattern as sensory neurones, however two distinct groups are present both of which express high levels of γ-synuclein. We believe that our investigation is robust and the findings provide an accurate picture of γ-synuclein expression in murine sensory and motoneurones. It is conceivable then, that γ-synuclein is interacting with different proteins or performing different functions in these two distinct populations, although it must be remembered these techniques only allow us a snapshot of the behaviour of the molecule.

5.2.2 Localisation of γ-synuclein in the *substantia nigra*

High levels of γ-synuclein mRNA expression have been previously reported in the *substantia nigra* (SN) (Lavedan et al., 1998a; Abeliovich et al., 2000).

Thus, we investigated the expression of γ-synuclein protein in the SN to determine whether or not these cells also displayed a change in sub-cellular localisation of γ-synuclein.

We saw the characteristic embryonic pattern of intracellular distribution at E15, i.e. both cell bodies and axons were stained positive for γ-synuclein. However, as in motoneurones, the staining of the perikarya was replaced by dotted neuropil staining by E18 with axonal staining persisting. The term neuropil is not ideal to use as it is quite non-descript, however its definition "A dense intricate feltwork of interwoven fine glial processes, fibrils, synaptic terminals, axons, and dendrites interspersed among the nerve cells in the grey matter of the central nervous system" makes it the most appropriate term to describe our observations. By P2 a reliable distinction between the SN and the ventral tegmental area (VTA) was possible and both the SN and VTA were found to display the same pattern of γ-synuclein expression.

These findings support those reported by Galvin et al. who observed a similar change in intracellular localisation of γ-synuclein in human embryos (Galvin et al., 2001b). Significantly though, a restricted number of neurones in adult animals did display γ-synuclein expression in the perikarya echoing the pattern seen in the cells of the facial nucleus. While not revealing any further information about the exact function of the synucleins, these findings do support the argument that although their expression begins during development and maturation of synapses, synucleins may

have a different function in maintaining normal synaptic function after development has ceased.

The striatum, which receives projections from the SN, also displays this neuropil staining suggesting that axonal and presynaptic expression of γ-synuclein is maintained in adult animals.

Once again these findings are in direct contrast to those of Li and colleagues who reported high levels of γ-synuclein staining in the cell bodies of the SN, both the *pars reticulata* and *pars compacta* (SNpc) (Li et al., 2002). Interestingly however we did observe positive staining in the perikarya in the cells of the red nucleus, in the same region as the SN, confirming the observation made by Li et al. This provided a useful internal positive control, validating our immunostaining methods.

We have proved the specificity of our anti-γ-synuclein antibody, utilising $\gamma^{-/-}$ mouse tissue and with no primary antibody assays serving as a negative control. We again suggest a reason for the apparent discrepancy between our expression profile and that of Li et al. (2002). Only in the arcuate nucleus of the hypothalamus, did Li et al. detect γ-synuclein as the sole synuclein present in that structure but with no verification of antibody specificity (Li et al., 2002). Thus the findings presented here give a more accurate analysis of γ-synuclein expression in the murine SN.

5.3 Synuclein null mutant mice

5.3.1 γ-synuclein null mutant mice

The generation of a true γ-synuclein null mutant was critical for studying the normal function(s) of γ-synuclein. This allowed us to investigate the hypothesis that γ-synuclein has an important role in the development and correct functioning of the

neuronal populations in which it is expressed. If this was the case, then its absence could lead to developmental and physiological abnormalities. It is important to acknowledge the fact that null mutant models are limited in their usefulness as they can only hint at a protein's endogenous function.

The successful generation of a γ-synuclein null mutant mouse strain has been discussed in detail previously and the absence of the wild-type allele, mRNA and protein in these animals has been confirmed by polymerase chain reaction, Northern and Western blotting respectively.

The colony was backcrossed onto a pure C57Bl6/J background prior to detailed characterisation of the mutant strain. The C57Bl6 animals used were sourced from Charles River, not Harlan. This is significant as a spontaneous α-synuclein null mutation was discovered in a strain of C57Bl6/S mice propagated in the UK Harlan facility (Specht and Schoepfer, 2001).

The γ-synuclein null mutant mice were viable and fertile and displayed no observable phenotype. A survey of gross brain morphology also showed that there were no phenotypic differences (Dr O. Schmidt, personal communication).

Adult γ-synuclein null mutant mice weighed the same as their wild-type littermates and displayed identical levels of activity in an open field camera, indicating that there was no obvious motor/perambulatory dysfunction. This test was applied during daylight hours at the same time. As mice are naturally more active at night, this would have been a more appropriate time to study this behaviour.

These observations echo those noted in four assessments of independently produced α-synuclein null mutant mice which also displayed no gross phenotypic abnormalities (Abeliovich et al., 2000; Specht and Schoepfer., 2001; Cabin et al.,

2002; Schluter et al., 2003). All but the mice described by Specht and Schoepfer, were created via homologous recombination as our γ-synuclein null mutant has been, while their mutant animal arose spontaneously as a result of commercial inbreeding. Specht and Schoepfer have concluded that as no significant increase in β- or γ-synuclein was detected via immunoblotting in these animals, compensation does not occur within the family. This is contradictory to our own findings and without looking in greater detail at the distribution of the remaining proteins and other combinations of synuclein null mutant I feel it is not possible to make such a statement.

Interestingly, it has recently been reported that $\beta^{-/-}$ and $\alpha^{-/-}/\beta^{-/-}$null mutant mice produced by Thomas Sudhof and colleagues also show no obvious phenotype (Chandra, Fornai, Kwon, Yazdani, Atasoy, Liu, Hammer, Battaglia, German, Castillo and Sudhof, 2004).

5.3.2 α-synuclein null mutant mice

The mice gifted to us by Prof A. Rosenthal (Genentech) were described as viable and fertile, exhibiting intact brain architecture and possessing a normal complement of dopaminergic cell bodies, fibres and synapses (Abeliovich et al., 2000). However, they exhibited an increased release of dopamine from nigrostriatal terminals in response to paired stimuli that could be mimicked by elevated Ca^{2+} (Abeliovich et al., 2000). These mice were of mixed 129sv/j / C57Bl6 background and experimental animals were only the F2 generation. These findings therefore may not be accurate as inter-strain differences can introduce unknown genetic variables making any comparisons unsound.

The α-synuclein null mutant mice were backcrossed onto the same pure C57Bl6 background as our γ-synuclein null mutant mice prior to any experimental use. With these strains of null mutant mice we could directly compare any effects of the absence of both α- and γ-synuclein. In addition, these true C57Bl6 single mutant mice were interbred to create double heterozygotes. These heterozygous littermates were then interbred to create a strain of $\alpha^{-/-}/\gamma^{-/-}$ -synuclein double mutant mice. This was a lengthy procedure as mice are not sexually mature until 5-6 weeks of age. It takes two months for each generation to pass and our experimental animals were at least the F6 generation. Therefore experimental double null mutant animals were not available for use in every part of this study.

5.4 Assay of sensory neurone populations

We have shown that the sensory neurones of the TG and DRG express high levels of γ-synuclein. The assessment of the number of sensory neurones in the DRG and TG of postnatal mice using Cresyl fast violet stained paraffin sections revealed no statistically significant differences in either the L6 DRG or TG in $\alpha^{-/-}$ and $\gamma^{-/-}$ mice as compared to their wild-type littermates. Interestingly, in a parallel study in the laboratory, no statistically significant differences were observed in the populations of cranial motoneurones (Ninkina et al., 2003).

These two different populations display a crucial difference in their expression pattern i.e. the shift in compartmentalisation seen in the majority of motoneurones during the peri-natal period discussed above. Despite high levels of expression and differences in sub-cellular localisation, no effects on these populations were observed in the absence of either α- or γ-synuclein (Ninkina et al., 2003). Thus, the absence of

either α- or γ-synuclein is not sufficient to disrupt the key developmental processes of neuronal migration, proliferation, differentiation or apoptosis in these studied ganglia.

However until all three synucleins are deleted and in each permutation, the true relationship between these proteins and development will not be revealed. The highly homologous nature of these proteins and their developmentally regulated expression strongly suggest a role lies within these processes. The absence of these proteins may only become detrimental to neuronal survival in old age, explaining their involvement in neurodegeneration. This would be of interest for future studies.

5.5 Survival of synuclein-deficient neurones in primary culture

It has been shown previously that certain types of cells, most notably dopaminergic cells, with modified synuclein expression, in particular α-synuclein over-expression, have normal survival characteristics under optimal culture conditions but are substantially more susceptible to stresses (Ostrerova-Golts et al., 2000; Petrucelli et al., 2002). Here we show that under optimal culture conditions (i.e. with NGF) there were no observable differences in the survival of neurones from γ-synuclein null mutant mice. We next tested the hypothesis that the absence of either α- or γ-synuclein may render cultured trigeminal neurones more susceptible to neurotoxic insult. We observed that both α- and γ-synuclein-deficient neurones survived equally poorly as cells taken from wild-type mice in the absence of NGF from the culture media.

It has been proposed that α-synuclein may be intrinsically neurotoxic as over-expression of mutated and endogenous α-synuclein has been shown to cause cell

death in culture (Saha et al., 2000; Zhou et al., 2000). These findings gave rise to the hypothesis that β- and γ-synuclein may act synergistically with α- protecting the cell from the toxic effects of α-synuclein. If this was the case in TG neurones, one might expect to have seen an increased level of cell survival in its absence. Conversely, in the absence of the negative regulation of γ-synuclein, the neurotoxic α-synuclein may have led to decreased survival under optimal conditions in γ-synuclein null mutant neurones.

The sensitivity of γ-synuclein null mutant neurones to direct neurotoxic insult was tested further. Proteosome inhibitors, metal ions, mutagens and JNK pathway inhibitors were all applied to cultures. Neurones from γ-synuclein null mutant mice displayed equivalent sensitivity to the toxic effects of these drugs as wild-type neurones.

We have shown previously that there are high levels of synuclein expression in sensory neuronal populations hence synucleins are likely to have a role in the normal function and development of these cells. However, disruption of the intracellular balance of synucleins did not lead to an alteration in sensitivity to neurotoxic insult in culture, which may indicate that synucleins are not essential to the processes which support neuronal survival. Indeed, the overlapping expression of these proteins could suggest that functional redundancy within this family exists, but the production of a triple synuclein null mutant would be necessary to answer this question.

The studies indicating that increased α-synuclein expression in dopaminergic cells in vitro causes neurotoxicity may not have revealed the true picture as it has recently been shown that glial derived trophic support in the form of glial derived

neurotrophic factor (GDNF), is critical for normal dopaminergic neuronal survival (Barroso-Chinea, Cruz-Muros, Aymerich, Rodriguez-Diaz, Afonso-Oramas, Lanciego and Gonzalez-Hernandez, 2005). This factor was not taken into account in the culture of DA neurones in either study, hence a decreased level of neuronal survival may have been exacerbated by the lack of trophic support coupled with the increased levels of α-synuclein present (Ostrerova-Golts et al., 2000; Petrucelli et al., 2002).

5.6 Developmental deficit of midbrain dopaminergic neurones

Mesencephalic dopaminergic neurones have been shown to express high levels of γ-synuclein mRNA and have also been described as the most vulnerable population to changes in the balance of synuclein expression (Lavedan et al., 1998a; Abeliovich et al., 2000; Hashimoto et al., 2001; Kruger and Schulz, 2002; Dev, Hofele, Barbieri, Buchman and van der Putten, 2003). With this in mind, we expanded our studies and assayed the dopaminergic neuronal populations of the SN at E18, P5 and in adult γ-synuclein null mutant mice. Total TH positive neurone number was taken, counting SN and the ventral tegmental area (VTA) as distinction between the embryonic SN and VTA was not possible. These investigations revealed a 15-20% deficit in the number of TH-positive neurones arising between E18 and P5, persisting at the same level into adulthood. Significantly, this deficit arises during the same peri-natal period as the shift in compartmentalisation occurring in motor and SN neurones. This loss of neurones occurs at approximately the same time as the major period of developmental apoptosis within the SN. This loss is biphasic in nature, peaking at P2 followed by a smaller peak at P14 (Burke, 2003). This cell death is regulated *in vivo*

by feedback from the target field, namely the striatum (Burke, 2003). While the controlling growth factor supplying these growing neurones with support is unknown, current evidence suggests that GDNF has a major role in preventing apoptosis during this period (Burke, 2003).

Our findings suggest that in the absence of γ-synuclein, mesencephalic dopaminergic (DA) neurones could have a reduced ability to utilise GDNF-derived neurotrophic support and hence are less able to survive this period of cell death. Conversely, alterations in the balance of synuclein proteins may make these cells more susceptible to neurotoxic insult.

To investigate this neuronal deficit and apportion it correctly to either the SN or the VTA, a second study was undertaken in adult animals. It was at this point in the study that α-/γ-synuclein double null mutant mice ($\alpha^{-/-}/\gamma^{-/-}$) became available for use. TH positive neurones were counted as previously described in the SN and VTA and this revealed not only that this deficit was specific to the SN, but it was present in $\alpha^{-/-}$ and $\alpha^{-/-}/\gamma^{-/-}$ mutant mice in equal measure. As no cumulative effects of the double mutation on neuronal number were seen, it is reasonable to hypothesise that a compensatory mechanism exists to limit the effects of their absence. β-synuclein therefore becomes a candidate for this function due to its homology with the other synuclein proteins and their overlapping expression pattern. These data cast doubt on the hypothesis that γ-synuclein acts as a negative regulator of the toxic α-synuclein as no further losses are seen in the absence of γ-synuclein and no recovery to wild-type levels was observed in $\alpha^{-/-}$ mice. It is possible that a distinct neuronal sub-population exists within the SNpc that are particularly vulnerable to a disruption in synuclein balance.

5.7 Basic motor function

In light of the neuronal deficit displayed by all three null mutant animals in the SNpc, a structure associated with motor behaviour, we tested these animals on a rotorod to look for any signs of impaired motor function. Two tests were carried out, one at a constant speed of 24 rpm and another accelerating from 0 to 40 rpm over the 5 minute duration of the test. No statistically significant differences were observed between any mutant groups and their wild-type littermates. It is likely that this deficit was insufficient to impact striatal DA levels to the extent which would precipitate a disturbance in motor control. In Parkinson's disease for example, clinical signs relating to the loss of motor control become apparent following a 70-80% reduction in striatal dopamine levels precipitated by a 40% reduction in mesencephalic dopaminergic neurones (Bezard et al., 2001). The neuronal deficit we report herein would not necessarily be great enough to precipitate a reduction in motor performance that could be detected using these methods. However as part of the overall phenotypic characterisation of these null mutants we felt it was important to test the animals as there may be other abnormalities leading to motor effects that were overlooked. The tests applied were as discriminating as the available equipment would allow.

5.8 Assaying striatal dopamine

The levels of striatal dopamine and its metabolites 5-hydroxyindolacetic acid, 3,4-dihydroxyphenylacetic acid and homovanillic acid were assayed using HPLC analysis, to detect whether or not the reduction in dopaminergic neurones seen in the SNpc translated into a reduction in striatal dopamine. No statistically significant

differences were observed between the three null mutant groups and their wild-type littermates. This was not unexpected as no disturbance of motor function was observed. These findings are in contrast to observations made in $\alpha^{-/-}/\beta^{-/-}$ animals in which, a 20% decrease in striatal dopamine levels were recorded, while no differences were seen in single mutant animals (Chandra et al., 2004). Other changes in this model included selective alterations in the levels of signalling and vesicular associated proteins; complexins and 14-3-3 proteins (Chandra et al., 2004). It has been proposed that these changes in vesicular proteins are the result of compensation for the loss of synucleins, due perhaps to a functional relationship between synucleins and these molecules which may exist *in vivo*. This could result from either the molecules acting in the same pathways or possibly direct interactions between these proteins (Chandra et al., 2004).

5.9 Compensatory changes in synuclein expression

It has been hypothesised previously that due to the high degree of homology shared by the synuclein proteins there may be a functional redundancy in the absence of one or more of these proteins. Here, we show significant evidence that supports this hypothesis, whereby all three of our synuclein null mutants displayed an increase in β-synuclein expression. This increase was similar in all strains i.e. no greater increase was seen in the absence of either α- and γ-synuclein. A 50% increase in γ-synuclein has also been reported in $\alpha^{-/-}/\beta^{-/-}$ animals lending greater support to this hypothesis (Chandra et al., 2004). Together these findings suggest that the synucleins are of great importance to normal neurodevelopment of certain populations. Hence, this compensatory mechanism has evolved to protect the endogenous physiological

functioning of the proteins which are still unknown. To further test this hypothesis siRNA techniques could be employed in different ratios in the different null mutants to attempt to exacerbate and rescue neuronal loss. Dopaminergic neurone culture would be an ideal model system to test cell survival under these conditions and would take experiments away from snapshots to a wider picture. A conventional β-synuclein knock-in to exacerbate the overproduction in the existing null mutant cohorts may produce a rescue of the dopaminergic neurones of the SNpc, which would strongly indicate that redundancy exists between the synucleins and their involvement in normal cell survival. A detailed investigation of the localisation of β-synuclein, using a highly specific antibody must be undertaken. Only then could any changes in compartmentalisation be identified. This may occur concurrently with the increased level of expression seen in our null mutants as potentially it may be replacing the function of the missing proteins.

5.10 Sensitivity of mesencephalic dopaminergic neurones to MPTP

MPTP is a potent dopaminergic specific neurotoxin which is widely used to create models of Parkinson's disease in rodents and primates. It is metabolised into the initial two-electron product 1-methyl-4-phenylpyridinium ($MPDP^+$) by type B monoamine oxidase (EC1.4.3.4, MAO-B) (Ramsay and Singer, 1986). In a further oxidation, the final four-electron oxidation product, the actual toxic molecule, 1-methyl-4-phenylpyridinium (MPP^+), is produced. The toxic intracellular accumulation of MPP^+ occurs as it is a high affinity substrate for the dopamine transporter (DAT) with which α-synuclein can interact (Javitch et al., 1985; Lee et al., 2001). The structural similarity between MPP^+ and dopamine is the reason

behind the specificity of the neurotoxin. Having been selectively taken up into the dopaminergic neurone, MPP^+ selectively inhibits NADH-ubiquinone oxioreductase (Complex I) of the mitochondrial respiratory chain. This inhibition leads to a failure of ATP production and hence cell death (Chan et al., 1991).

We tested the sensitivity of our synuclein null mutant mice to MPTP. Wild-type animals showed the expected 40% reduction in dopaminergic neurones for our chosen MPTP dose regimen when compared to vehicle-injected controls. In contrast, all three null mutant groups showed complete insensitivity to MPTP in comparison with a vehicle-injected control group, with no observable changes in neuronal number. Independent studies have confirmed that other null mutations for α-synuclein provide some degree of protection from the toxic effects of MPTP, with a reduced decrease in striatal dopamine being recorded (Schluter et al., 2003; Drolet et al., 2004). In the initial report of this protective effect it was proposed that in the absence of α-synuclein, MPTP could not inhibit Complex I thus eliminating the toxic potential (Dauer et al., 2002). Commercially available spontaneous α-synuclein deficient mice display no such reduction in sensitivity (Schluter et al., 2003). However, these animals lack several other genes that could easily account for this insensitivity. These data, ours included, suggest that MPTP toxicity is not directly coupled to any interactions of synuclein with proteins that are involved in mediating this toxic effect but that these proteins do influence the mechanism in some mouse strains. Stimulation of expression, post-translational modification and aggregation of α-synuclein in the neurones of the SNpc by MPTP is well documented, but it is less clear how these correlate with neuronal death (Kowall et al., 2000; Vila et al., 2000; Przedborski et al., 2001; Meredith et al., 2000; Kuhn et al., 2003). Studies of

dopaminergic neurones that over-express different forms of human α-synuclein produced contradictory results with either increased (Richfield et al., 2002) or unaltered (Rathke-Hartlieb et al., 2001; Dong et al., 2002) sensitivity to MPTP toxicity. Results showing a resistance to MPTP toxicity are more consistent for mice with targeted inactivation of the α-synuclein gene. The neuroprotective effect of β-synuclein over-expression has recently been demonstrated in *in vitro* experimental systems (da Costa, Masliah and Checler, 2003). In cultured neurones cells the over-expression of β-synuclein has been shown to inhibit the expression of p53 thereby protecting cells from staurospaurine-induced cell death. It is feasible therefore to suggest that any compensatory increases in β-synuclein levels in midbrain neurones that we have shown here could render these neurones less sensitive to certain neurotoxic insults by preventing the cell entering apoptosis. Again, it would be interesting to examine the sensitivity of these neurones in the absence of all three synucleins and to see if this insensitivity remains in the absence of the potential compensatory effect of β-synuclein. The same experimental cohort was tested prior to tissue collection using the accelerating rotorod trial described previously, having been given training prior to any substance administration. This procedure was to ensure that no deleterious effects on motor performance were produced by the drug acting on other neuronal populations concerned with controlling motor function. As no neuronal deficits were observed within the SN, no significant differences were expected. Thus it was not surprising that no differences were observed between either drug or control injected or genotypic groups.

5.11 Conclusions

Several synuclein transgenic models, including our own have demonstrated that the absence of α-, β- or γ-synuclein individually or α-/β-, α-/γ- combined, does not lead to any gross changes in the morphology or physiology of the nervous system. We have shown here using null mutant models that deficits arise in specific neuronal populations in the absence of certain synucleins, indicating that they play a role in normal neurodevelopment. Our findings do not directly support the widely held hypothesis that α-synuclein is intrinsically neurotoxic as we did not observe any increased neuronal survival in its absence *in vitro* or *in vivo*. However our study has focused mainly on developing and early adult animals in an attempt to gain information relating to their normal function. As the pathologies associated with the synuclein proteins tend to arise at later ages, perhaps later time points would have been more suitable to challenge these synuclein deficient populations in our experimental models. Perhaps introducing tests of learning and memory may also yield results, as tests currently available for motor behaviours are not sensitive enough to detect phenotypes in our null mutant cohorts, if indeed they exist.

The weight of evidence however does still indicate that fibrillated α-synuclein is cytotoxic under certain physiological conditions. The role of β-synuclein must be investigated further as it has been shown to act synergistically with α-synuclein. Also, as we have shown here in the absence of α- and γ-synuclein, the expression of β-synuclein is increased, perhaps to maintain some endogenous function of the synucleins, which still eludes us.

The production and characterisation of a complete synuclein null mutant is underway in collaboration between Prof. V. Buchman and Prof. T. Sudhof. It will be interesting

to learn whether the changes we report here are exacerbated in the absence of all three synucleins and hopefully more concrete evidence will emerge as to the mechanisms in which synucleins are involved. As mentioned above the insensitivity to MPTP we report, may be lost in the absence of all three proteins, which should conclusively prove that some functional redundancy does exist between the molecules. Also, the dopaminergic cell loss in the SNpc may also be exacerbated by the complete loss of synucleins. Culturing of dopaminergic neurones taken from the transgenic cohorts would provide a useful tool as they could be grown in combination with different synuclein siRNA to knock-down the remaining synucleins in the different null mutants. The effects of these manipulations would hopefully demonstrate the differing contributions the synucleins make towards dopaminergic cell survival. Very recently it has been shown that null mutant mice lacking trkB and trkC, the receptors for dopaminergic growth factors brain derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) respectively, also exhibit an approximate 20% reduction in the number of TH positive neurones in the SNpc in addition to increased expression of α-synuclein in the remaining cells of aged mice (von Bohlen, Halbach, Minichiello and Unsicker, 2005). If this increase in α-synuclein expression is a direct result of reduced trophic support for the remaining cells then it implicates the synucleins in mediating trkB and trkC signalling and would help to explain the developmental deficit in midbrain dopaminergic neurones we report here. However the authors of this paper did not comment on this possibility and propose that α-synuclein is accumulating as cells begin the deterioration that occurs with age.

In summary, the evidence for synergistic action between the three synuclein proteins continues to mount yet the vast majority of studies overlook the existence and possible significance of β- and γ-synuclein. I feel that this may be a potential cause for some of the conflicting data that has arisen in this field of study.

The precise role of this protein family is still unclear, with recent data implicating them in the regulation of cell survival. Whether they are cytotoxic, protective in nature or function in synergy as a distinct regulator of cell survival will not be determined until the details of their endogenous function are elucidated in isolation from one and other.

Chapter 6: Bibliography

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APPENDIX

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Neurons Expressing the Highest Levels of γ-Synuclein Are Unaffected by Targeted Inactivation of the Gene

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Homologous recombination in ES cells was employed to generate mice with targeted deletion of the first three exons of the γ-*synuclein* gene. Complete inactivation of gene expression in null mutant mice was confirmed on the mRNA and protein levels. Null mutant mice are viable, are fertile, and do not display evident phenotypical abnormalities. The effects of γ-synuclein deficiency on motor and peripheral sensory neurons were studied by various methods in vivo and in vitro. These two types of neurons were selected because they both express high levels of γ-synuclein from the early stages of mouse embryonic development but later in the development they display different patterns of intracellular compartmentalization of the protein. We found no difference in the number of neurons between wild-type and null mutant animals in several brain stem motor nuclei, in lumbar dorsal root ganglia, and in the trigeminal ganglion. The survival of γ-synuclein-deficient trigeminal neurons in various culture conditions was not different from that of wild-type neurons. There was no difference in the numbers of myelinated and nonmyelinated fibers in the saphenous nerves of these animals, and sensory reflex thresholds were also intact in γ-synuclein null mutant mice. Nerve injury led to similar changes in sensory function in wild-type and mutant mice. Taken together, our data suggest that like α-synuclein, γ-synuclein is dispensable for the development and function of the nervous system.

Several neurodegenerative diseases have been recently coalesced into a distinct group named synucleinopathies (12, 16, 20, 53). Although they are diverse in symptoms and clinical signs, these diseases share a common histopathological feature, i.e., formation of large intracellular inclusions whose principal component is an aggregated small protein, α-synuclein. Neither the normal cellular function of α-synuclein nor the exact mechanism of its involvement in neurodegeneration is clearly understood; possible scenarios are discussed in many recent reviews (see, for example, references 10, 28, 33, 34, and 43). Even less clear are the normal functions and roles in neurodegeneration of the other two members of the synuclein family. Both β-synuclein/PNP14 (24, 35) and γ-synuclein/BCSG1/persyn (7, 26, 29) have a very high degree of amino acid similarity with α-synuclein within the N-terminal KTK repeat region of the protein molecule, and this is reflected in such common features of synucleins as a native unfolded state in physiological solutions, reversible binding to lipid vesicles, and localization in presynaptic terminals (13, 25, 31). However, the C-terminal regions of synucleins, although all highly acidic, are rather different (7, 29, 52). It is perhaps this structural diversity that leads to differences in the behavior of synucleins in vitro and in various in vivo model systems. Consistent with the finding that β-synuclein and γ-synuclein are much less fibrillogenic than α-synuclein (4, 47, 55), aggregates of these two proteins are not constituents of Lewy bodies or other histopathological hallmarks of synucleinopathies, although abnormal β- and γ-synuclein-positive structures have been observed in several cases (15, 17, 49). Recent in vitro studies have also shown that both β- and γ-synuclein are able to inhibit fibrillation of α-synuclein (40, 55). In transgenic mice overexpression of β-synuclein reduces the severity of neurodegenerative alterations and the number of α-synuclein-positive interneuronal inclusions caused by α-synuclein overexpression (23). Changes of expression of all three synucleins in brain areas affected in neurodegenerative diseases have been reported (44). Previously we demonstrated that overexpression of α-synuclein, but not γ-synuclein, kills sensory neurons in primary cultures (6, 45). Moreover, it has been shown that γ-synuclein is able to block JNK signaling, a pathway whose activation is commonly associated with induction of apoptosis (39). These observations suggest that the correct balance of synucleins might be important for survival of at least some populations of neurons and that decreased expression of γ-synuclein might have a proapoptotic effect. The obvious way to investigate this is to assess whether the absence of γ-synuclein affects neurons that normally express these two proteins. In different vertebrate species, high levels of γ-synuclein mRNA are detected from the early stages of embryonic development in two neuronal populations, motoneurons and peripheral sensory neurons (7, 52), and α-synuclein is also expressed in these neurons (18, 32, 52; our unpublished observations). Moreover, in transgenic mice overexpression of

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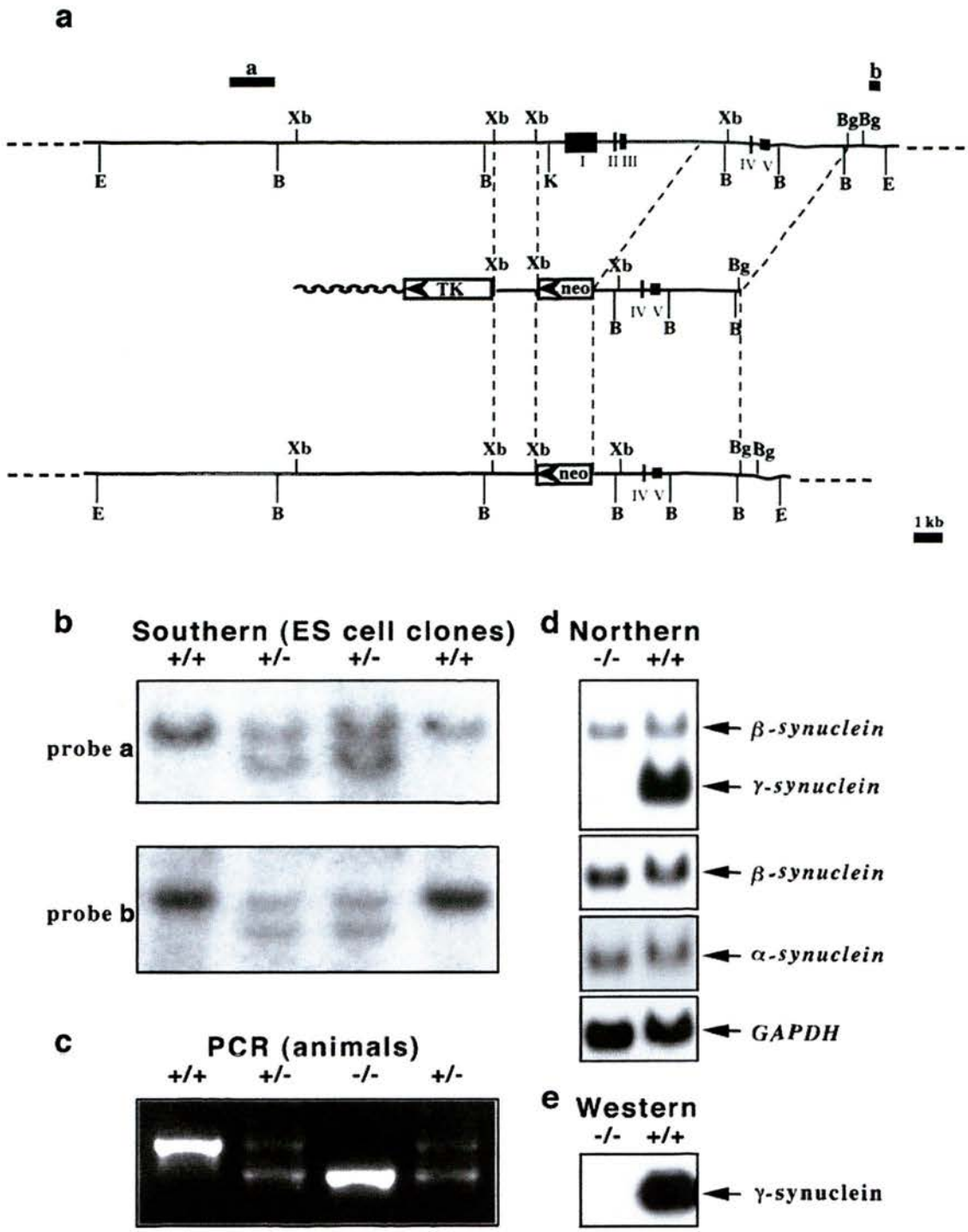


FIG. 1. Targeted inactivation of the mouse γ-*synuclein* gene. (a) Scheme for deletion of exons I, II, and III and promoter region of the mouse γ-*synuclein* gene by homologous recombination. The organizations of the wild-type genomic locus (top), targeting vector (middle), and resulting knockout locus (bottom) are shown. Restriction endonuclease sites: E, *Eco*RI; B, *Bam*HI; Xb, *Xba*I; K, *Kpn*I; Bg, *Bgl*II. Hybridization probes a and b, which were used for the analysis of homologous recombination, are also shown. (b) Examples of analysis of homologous recombination in ES cell lines by Southern hybridization. DNAs from four neomycin-resistant ES cell lines were digested with *Eco*RI and hybridized with either probe a or probe b. Only a 20-kb wild-type band is revealed in two clones with random insertion of a *PGK-neo* cassette, and the homologous recombination in two other clones results in the appearance of a 17-kb band. Similar results were obtained when DNA was digested with *Bam*HI and hybridized with probe c. (c) Example of PCR-based genotyping of mice from a litter of two heterozygous parents. (d) Expression of mRNAs encoding members of the synuclein family in the retinas of wild-type and γ-synuclein null mutant mice. Results of Northern hybridization with a full-length mouse γ-*synuclein* cDNA probe, a mouse β-*synuclein*-specific probe, a mouse α-*synuclein*-specific probe, and a *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) probe are shown. Note that under the hybridization and washing conditions used, the γ-*synuclein* cDNA probe cross-hybridized with β-*synuclein* transcript (upper panel). High-stringency washes completely eradicated this hybridization signal, with no effect on hybridization with γ-*synuclein* transcript. (e) A Western blot of 10 μg of total spinal cord proteins of wild-type and γ-synuclein null mutant mice was probed with mouse γ-synuclein-specific SK23 antibody.

α-synuclein leads to pathological changes in spinal and brain stem motoneurons (19, 30, 56), suggesting that these neurons are susceptible to changes in the metabolism of synucleins. Therefore, we studied populations of motoneurons and peripheral sensory neurons in γ-synuclein null mutant mice, which we produced.

MATERIALS AND METHODS

Generation of *γ-synuclein* null mutant mice. To generate a targeting vector, a 1.2-kb *Xba*I-*Xba*I fragment from lambda genomic clone PS92 (2) was cloned as a short arm into an *Xba*I site of the pPNT vector (54) between *PGK-TK* and *PGK-neo* cassettes. The resulting plasmid was digested with *Sse*8387I, blunted with the Klenow fragment of DNA polymerase I, further digested with *Xho*I, and used as a vector for ligation with a long arm. DNA of lambda genomic clone PS91 (2) was digested with *Bgl*II, blunted with Klenow fragment of DNA polymerase I, and further digested with *Sal*I, and a 4-kb fragment was used as a long arm in our targeting vector (Fig. 1a). For electroporation of 129Ola mouse ES cells (clone E14Tg2a; a gift of A. Smith, Centre for Genome Research, University of Edinburgh), DNA of the resulting targeting vector was linearized by digestion with *Not*I. After electroporation and selection, G418- and ganciclovir-resistant ES cell clones were checked for correct homologous recombination by Southern hybridization analysis of *Eco*RI-digested (for probes a and b in Fig. 1a) or *Bam*HI-digested (for probe c) DNA. Suitable clones were injected into blastocysts of C57Bl6J mice (Charles River). All procedures involved in generation of mutant mice were carried out according to the United Kingdom Animals (Scientific Procedures) Act (1986) and other Home Office regulations under specific-pathogen-free conditions. The presence of a mutant allele in animals was checked by PCR analysis of DNA extracted from mouse tail biopsies. A common upstream primer (5′-AGTCCTGGCACCTCTAAGCA-3′) and primers specific for the wild-type allele (5′-GGGCTGATGTGTGGCTATCT-3′) and the *PGK-neo* cassette in the mutant allele (5′-GAAGAACGAGATCAGCAGCC-3′) were used for amplification. Forty cycles of 45 s at 95°C, 30 s at 56°C, and 60 s at 72°C were carried out. The presence of 480-bp (for the wild-type allele) and 397-bp (for the mutant allele) amplification products in the reaction mixture was checked by electrophoresis in a 1.5% agarose gel. Heterozygous animals were used for at least six further generations of backcrosses with C57BL6J mice before null mutant, wild-type, and heterozygous littermates were produced for further studies by breeding heterozygous males and females. The mutant mouse strain was registered in the Mouse Genome Information database under the official name (B6-TgHSNCG^{tm1VLB}).

α-*synuclein* null mutant mice. Generation of α-*synuclein* mutant mice was described previously (1). We backcrossed these mice with C57Bl6J mice for at least six generations before setting up intercrosses to produce null mutant, wild-type, and heterozygous littermates.

Expression studies. Extraction of RNA from mouse tissues, Northern blotting, and preparation of labeled probes were carried out as described previously (7, 36).

Affinity-purified polyclonal rabbit SK23 antibody generated against a C-terminal peptide of mouse γ-synuclein (persyn) was used at a 1:500 dilution for Western blotting and enhanced chemiluminescence detection of this protein in total cell lysates as described earlier (7, 36). For immunohistochemistry, adult mouse brains and embryonic day 12 (E12), E15, and E18 embryos were fixed in 4% paraformaldehyde–phosphate-buffered saline (PBS) or Carnoy's fixative (60% ethanol, 30% chloroform, 10% glacial acetic acid) at 4°C overnight following dehydration in alcohol series and embedding in paraffin blocks. Eight-micrometer-thick sections were cut with a Leica or Microm microtome and mounted on SuperFrost slides (BDH, Poole, United Kingdom) for conventional staining or on Gold Seal slides (Gold Seal Products, Portsmouth, N.H.) for immunostaining. The paraffin sections were cleared in xylene and rehydrated through a graded alcohol series. Endogenous peroxidase activity was quenched by incubating the slides in 3% H_2O in methanol for 20 min. After being washed with PBS, the tissues were blocked in 10% horse or goat serum and 0.4% Triton X-100 in PBS for 1 h at room temperature. Incubation with a 1:40 dilution of SK26 anti-mouse γ-synuclein antibody was carried out at 4°C overnight. Detection of immune complexes with biotinylated anti-rabbit antibody and avidin-peroxidase complex from the Vectastain ABC kit (Vector Laboratories, Peterborough, United Kingdom) and diaminobenzidine (Fast 3,3′-diaminobenzidine tablet sets; Sigma, St. Louis, Mo.) as a substrate was carried out according to the manufacturer's instructions. Motor nuclei were identified by staining of alternate sections with hematoxylin-eosin (Raymond A Lamb, London, United Kingdom) and a goat antibody against choline acetyltransferase (Chemicon, Temecula, Calif.) at a 1:100 dilution. In the latter case, anti-goat secondary antibody was used for detection.

Whole-mount immunofluorescence. Mouse triangularis sterni muscle was dissected in PBS and fixed in 4% paraformaldehyde–PBS for 20 min, followed by incubation with AlexaFluor 647-conjugated μ-bungarotoxin (5 μg/ml in PBS) for 30 min. After washing in PBS and blocking and permeabilization for 1 h in 1% bovine serum albumin–0.4% lysine–0.5% Triton X-100 in PBS, samples were incubated overnight with primary antibodies diluted in the same buffer at 4°C. Mouse monoclonal anti-neurofilament M (clone 2H3; Developmental Studies Hybridoma Bank, Iowa City, Iowa) and anti-synaptic vesicles (clone SV2; Developmental Studies Hybridoma Bank) antibodies were used at a 1:200 dilution, and rabbit polyclonal SK23 antibody was used at a 1:40 dilution. After several washes, incubation with 1:200-diluted secondary antibodies (fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulins and tetramethyl rhodamine isocyanate-conjugated goat anti-mouse immunoglobulins, both from Jackson ImmunoResearch Laboratories, West Grove, Pa.) was carried out for 3 h at room temperature, followed by extensive washing and mounting in Vectashield (Vector Laboratories, Burlingame, Calif.). Images were obtained with a Zeiss LSM 510/Axioplan 2 confocal microscope.

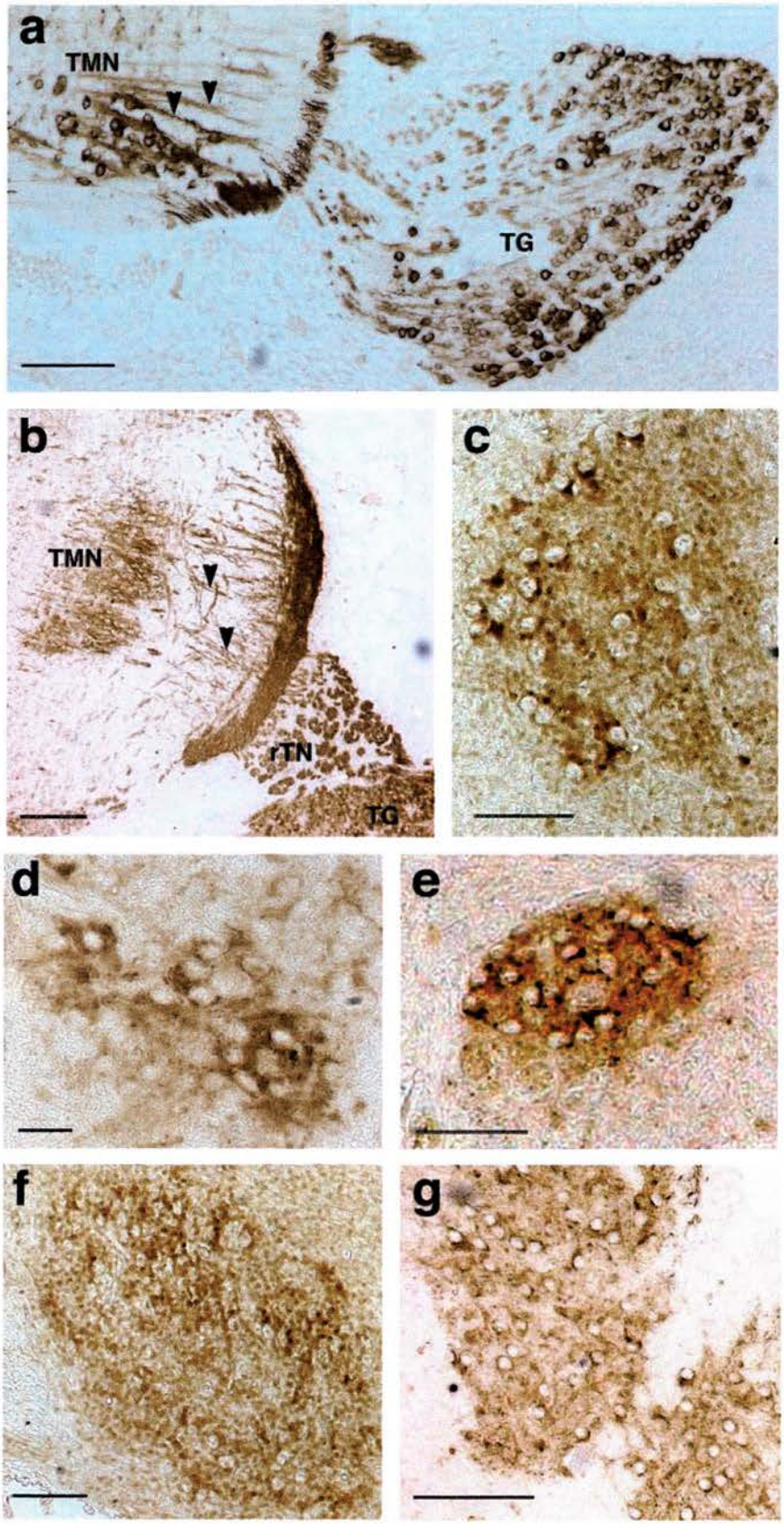
DRG neuron counts. The spinal columns from P2 mice were fixed and embedded as described above. Serial 8-μm-thick longitudinal sections were cut, mounted on SuperFrost slides (BDH), cleared in xylene, rehydrated through a graded alcohol series, and stained with 0.1% cresyl violet acetate (Sigma). Lumbar L6 dorsal root ganglia (DRG) were identified by using characteristic tissue landmarks on sections. Neurons were identified by virtue of the Nissl substance and their large, round, pale-stained nuclei (27). Neurons in the L6 DRG displaying a prominent nucleolus were counted on every eighth section. The neuronal number was quantified by using a digital stereology system that employs a combination of the optical dissector and volume fraction-Cavalieri methods (Kinetics Imaging, Bromborough, United Kingdom). All counts were carried out blindly by a person unaware of animal genotypes.

Motoneuron counts. Adult mouse brains were fixed and processed as described above for expression studies. The whole brain stem was sectioned into 8-μm-thick sections, which were mounted on SuperFrost slides. Rehydrated sections were stained in a 1% solution of neutral red (Raymond A Lamb) in distilled water for 30 min, followed by dehydration and mounting as described above. A stereological fractionator method (22), which allows estimation of the number of particles independent of the volume of the structure that the particles are part of and is not affected by tissue shrinkage during processing, was used to assess the number of motoneurons in five motor nuclei of the brain stem. Large (~20 μm in diameter) neurons displaying a prominent nucleolus were counted in total on every fifth section throughout the nucleus; the first section for counting was randomly chosen from the first five sections that included this nucleus.

Nerve fibers counts. Anesthetized animals were transcardially perfused with heparinized saline and then 2.5% glutaraldehyde–2% paraformaldehyde–0.1 M sodium cacodylate buffer (pH 7.3)–1 mM $CaCl_2$ for 5 min. The saphenous nerve was removed and fixed for 2 h in the same fixative, postfixed in OsO_4, and embedded in araldite. One-micrometer resin sections were prepared and stained with toluidine blue for light microscopy to measure the area of the nerve cross-section. Ultrathin 80-nm transverse sections were stained with uranyl acetate and lead citrate, mounted onto copper slot grids, and examined on a transmission electron microscope (Phillips BioTwin; FEI, Cambridge, United Kingdom). Myelinated A-fibers and unmyelinated C-fibers were identified and counted as described previously (57). The densities of both types of fibers were calculated after counting of 12 to 15 randomly chosen electron microscope images and multiplied by the area of the nerve cross-section.

Primary neuronal cultures. Cultures from trigeminal and superior cervical ganglia were prepared as described previously (42) and maintained in neurobasal medium with B27 complement (Invitrogen, Carlsbad, Calif.). The number of neurons attached to each culture dish within a 12- by 12-mm square was counted 3 h after plating and was taken as the initial number of neurons (100%). In experiments with proteasome inhibitors and metal ions, the initial count was carried out 24 h after plating. In all cases drugs were added to cultures immediately after the initial count. The number of surviving neurons in the same area was counted 24 and 48 h later and was expressed as a percentage of the initial count.

Behavioral tests. All experiments were carried out in accordance with the United Kingdom Animals (Scientific Procedures) Act (1986). Behavioral testing was carried out prior to surgery to establish a baseline for comparison to post-surgical values, as described in detail previously (5, 11). The procedure of chronic constriction injury (CCI) to the sciatic nerve was modified for mice from a procedure described previously for rats (3). In brief, adult male mice (90 to 120 g) were anaesthetized with sodium pentobarbital (Sagatal; Rhône Merieux,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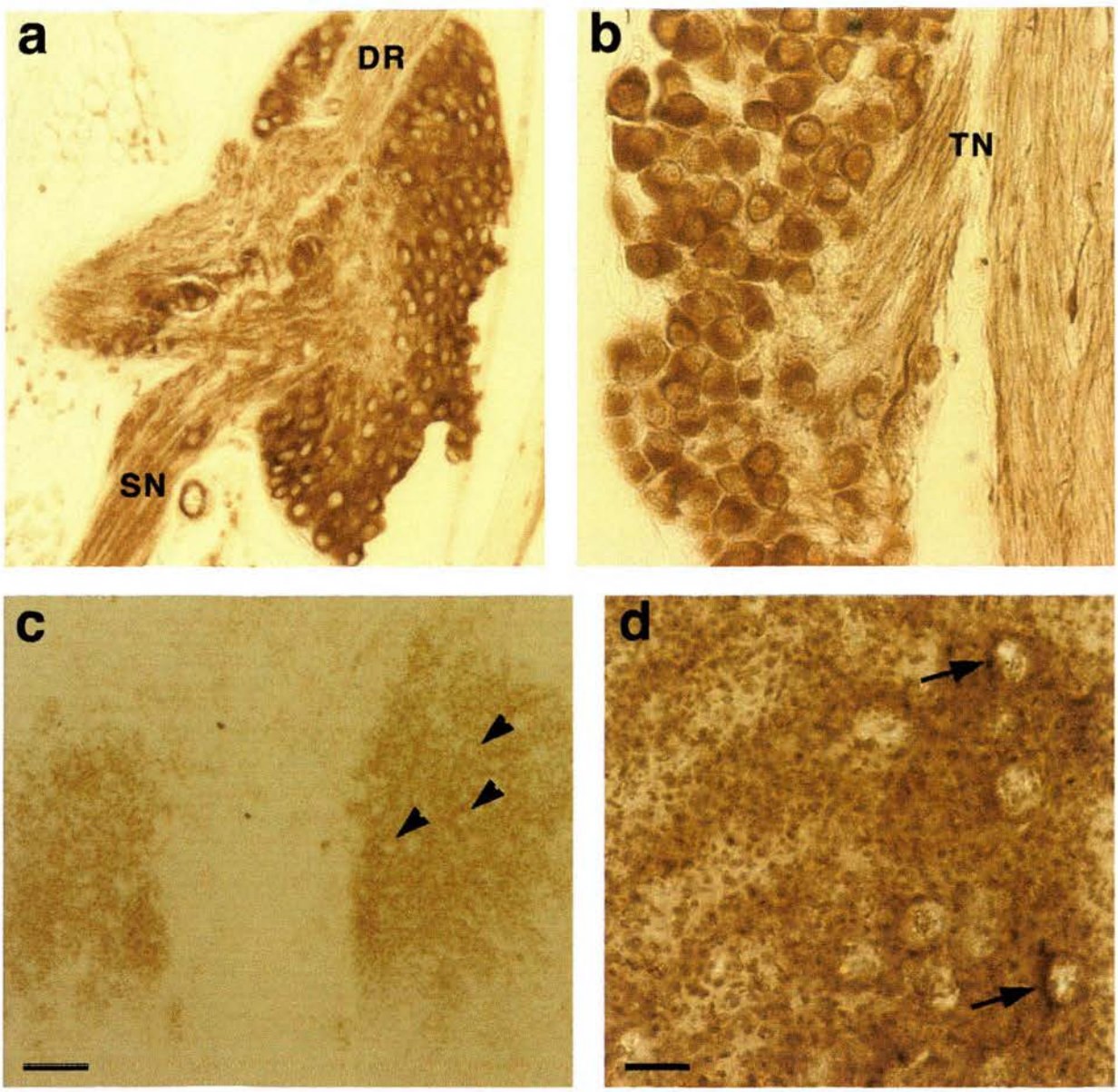
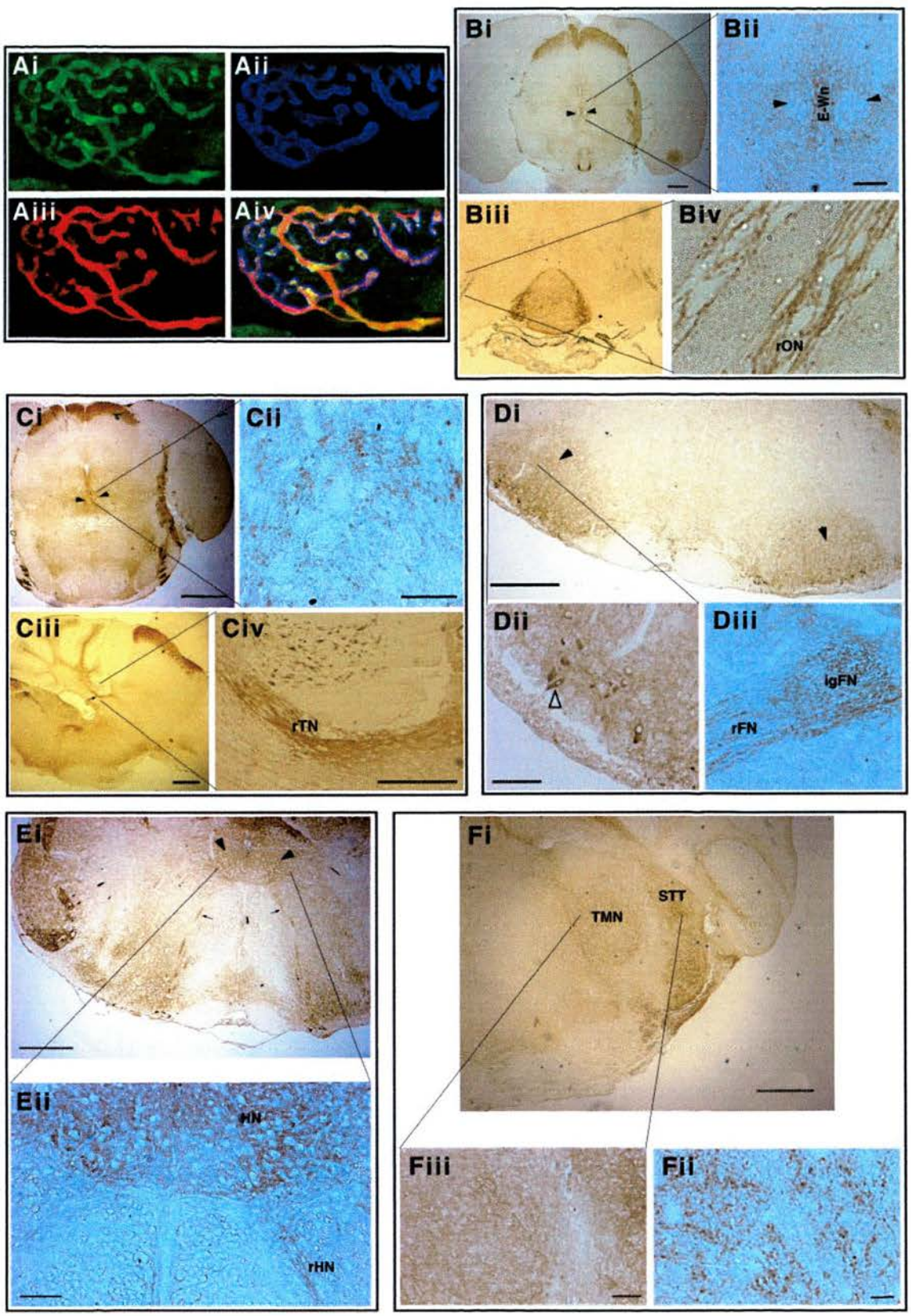


FIG. 3. γ-Synuclein in postnatal mouse sensory and motoneurons. γ-Synuclein is localized in the cytoplasm of cell bodies of mouse P2 DRG (a) and trigeminal ganglion (b) neurons as well as in nerve fibres in the dorsal root (DR), spinal nerve (SN), and trigeminal nerve (TN). In P2 oculomotor nucleus (c), only γ-synuclein-negative cell bodies (arrowheads) are seen on the background of neuropil staining, whereas in P2 trigeminal motor nucleus (d), cytoplasmic staining can be detected in some neurons (arrows). Bars, 100 μm (a and c) and 20 μm (b and d).

Essex, United Kingdom) (0.06 ml/100 g, intraperitoneally) supplemented with halothane-O_2 (Zeneca, Cheshire, United Kingdom). Under aseptic conditions, the right sciatic nerve was exposed proximal to the trifurcation, at mid-thigh level, and three chromic cat gut ligatures were tied to loosely constrict the nerve. The overlying muscle and skin were closed with sutures, and the animals were allowed to recover before reflex testing recommenced. Thermal hyperalgesia was monitored by using noxious radiant heat (30 to 55°C) (Hargreaves' thermal device; Linton Instruments, Diss, United Kingdom) applied to the mid-plantar glabrous surface of the hind paw. The withdrawal response latency was characterized as a brief paw flick, and a standard cutoff latency of 20 s prevented tissue damage. Mechanical allodynia was measured as the threshold for paw withdrawal in response to graded mechanical stimuli applied to the mid-plantar glabrous

FIG. 2. γ-Synuclein in embryonic mouse sensory and motoneurons. Anti-γ-synuclein staining of E12 (a) and E15 (b) trigeminal motor nuclei and trigeminal ganglia and of E18 trigeminal motor nucleus (c), E18 oculomotor nucleus (d), E18 trochlear nucleus (e), E18 facial nucleus (f), and E18 hypoglossal nucleus (g) is shown. All nuclei contain labeled neuronal cell bodies, but dotted neuropil stain becoming also obvious in all ganglia at E18. Arrowheads show γ-synuclein-positive axons of E12 and E15 motoneurons of the trigeminal motor nucleus (TMN). Sensory neurons of the trigeminal ganglion (TG) are also intensively stained, as is the root of the trigeminal nerve (rTN). Bars, 100 μm (a, c, e, and f), 200 μm (b and g), and 50 μm (d).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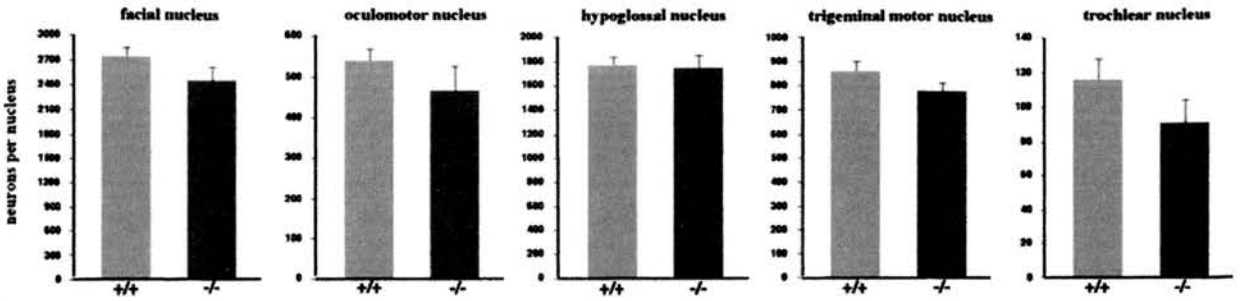

FIG. 5. Average total numbers of motoneurons in brain stem motor nuclei of wild-type (+/+) and γ-*synuclein* null mutant (-/-) adult mice. Means and standard errors from data obtained from analysis of at least 10 nuclei for each genotype are shown. Statistical analysis (two-tailed, unpaired Student's *t* test) showed no significant difference in cell numbers between the two genotypes for all nuclei ($P > 0.5$).

surface of the hind paw with calibrated von Frey filaments (Stoelting, Wood Dale, Ill.). The threshold was defined as the pressure (force per unit area) that caused foot withdrawal five times in every 10 applications, repeated at 1- to 2-s intervals. The pressure applied to the hind limb by the von Frey filaments is calibrated as force (millinewtons) divided by the area over which it is applied (square millimeters).

RESULTS

Generation of γ-*synuclein* null mutant mice. Mouse strains with a targeted deletion of the first three exons of the mouse γ-*synuclein* gene (Fig. 1) were generated from three independent ES cell clones as described in Materials and Methods. Two of these strains were used in this study, and because the results were identical, the experimental data obtained with both strains were combined. Mice lacking the γ-*synuclein* gene were viable and fertile and did not show any obvious abnormalities in development, behavior, or gross morphology of the nervous system (data not shown). Western blotting (Fig. 1e) and immunohistochemistry (not shown) with an antibody specific to the C-terminal peptide of mouse γ-synuclein showed a complete absence of the protein in all tissues of null mutant mice. Northern hybridization of RNAs from various tissues confirmed complete inactivation of γ-*synuclein* gene function in null mutant mice, which was not accompanied by compensatory increases of α- or β-*synuclein* mRNA levels (Fig. 1d and data not shown).

In the absence of an obvious phenotypic manifestation of the null mutant genotype in γ-synuclein knockout mice, we carried out comparative studies of several neuronal populations with wild-type and null mutant mice. Motoneurons and peripheral sensory neurons were chosen because both populations normally express high levels of γ-*synuclein* mRNA (7) but, as shown below, display different intracellular compartmentalization of γ-synuclein.

Developmental changes of γ-synuclein compartmentalization in motoneurons. Previously we have demonstrated by in situ hybridization high levels of γ-*synuclein* mRNA expression in spinal and cranial motoneurons from early stages of mouse and rat embryonic development (7). However, neither the abundance nor the intracellular localization of γ-synuclein protein in these neurons has been studied. Using a specific antibody which recognizes the C-terminal peptide of mouse γ-synuclein, we now demonstrated that the protein expression pattern follows the mRNA expression pattern but that dramatic changes in compartmentalization of γ-synuclein within motoneurons take place during postnatal development. Immunohistochemistry was performed on sagittal as well as coronal paraffin sections of mouse embryos of different developmental stages and adult mouse brains. To identify oculomotor, trochlear, facial, hypoglossal, and trigeminal motor nuclei, adjacent sections were stained with hematoxylin-eosin and an antibody against choline acetyltransferase, a motoneuron marker (see Materials and Methods). From the early stages (E12) and throughout the embryonic development, γ-synuclein is localized in both motoneuron cell bodies and axons (Fig. 2 and data not shown). However, at E18, in addition to cytoplasmic staining, a dotted neuropil staining became evident in all studied motor nuclei (Fig. 2c to g). The pattern is completely different in motor nuclei of postnatal, particularly adult, mouse brain.

FIG. 4. γ-Synuclein in adult mouse motoneurons. (A) γ-Synuclein in motor axons and nerve terminals at the neuromuscular synapse. Triple immunofluorescent staining of whole-mount preparations of mouse traingularis sterni muscle is shown. γ-Synuclein (Ai, green) is colocalized with neurofilaments in the axon and SV2 in presynaptic terminals (Aiii, red) but not with acetylcholine receptors on postsynaptic membrane (Aii, blue [stained with AlexaFluor 647-conjugated μ-bungarotoxin]). Three images are merged in panel Aiv. (B) γ-Synuclein in oculomotor nuclei (arrowheads) and the root of oculomotor nerve (rON). A higher magnification shows axonal staining in the nerve root (panel Biv), the absence of γ-synuclein in cell bodies of motoneurons, and positive staining of neuronal cell bodies in the median Edinger-Westphal nucleus (E-Wn, panel Bii). (C) γ-Synuclein in trochlear nuclei (arrowheads in panel Ci) and the root of trochlear nerve (arrow in panel Ciii and rTN in panel Civ). A higher magnification shows intense staining of motoneuron axons in the nerve (Civ) and only a dotted neuropil staining in the nucleus (Cii). (D) γ-Synuclein in facial nuclei (arrowheads) and the facial nerve. Axonal staining is evident in the internal genu (IgFN) and the root (rFN) of the nerve (panel Diii). A higher magnification reveals γ-synuclein in the cytoplasm of a few motoneurons (open arrowhead in panel Dii). (E) γ-Synuclein in hypoglossal nuclei (arrowheads in panel Ei and HN in panel Eii) and the root of hypoglossal nerve (rHN in panel Eii). A higher magnification shows positive staining of the nerve root and neuropil staining in the nucleus (Eii). (F) γ-Synuclein in the trigeminal motor nucleus (TMN) and the spinal trigeminal tract (STT). Neuronal cell bodies are not stained; only dotted neuropil staining is evident at the highest magnification (Fiii).

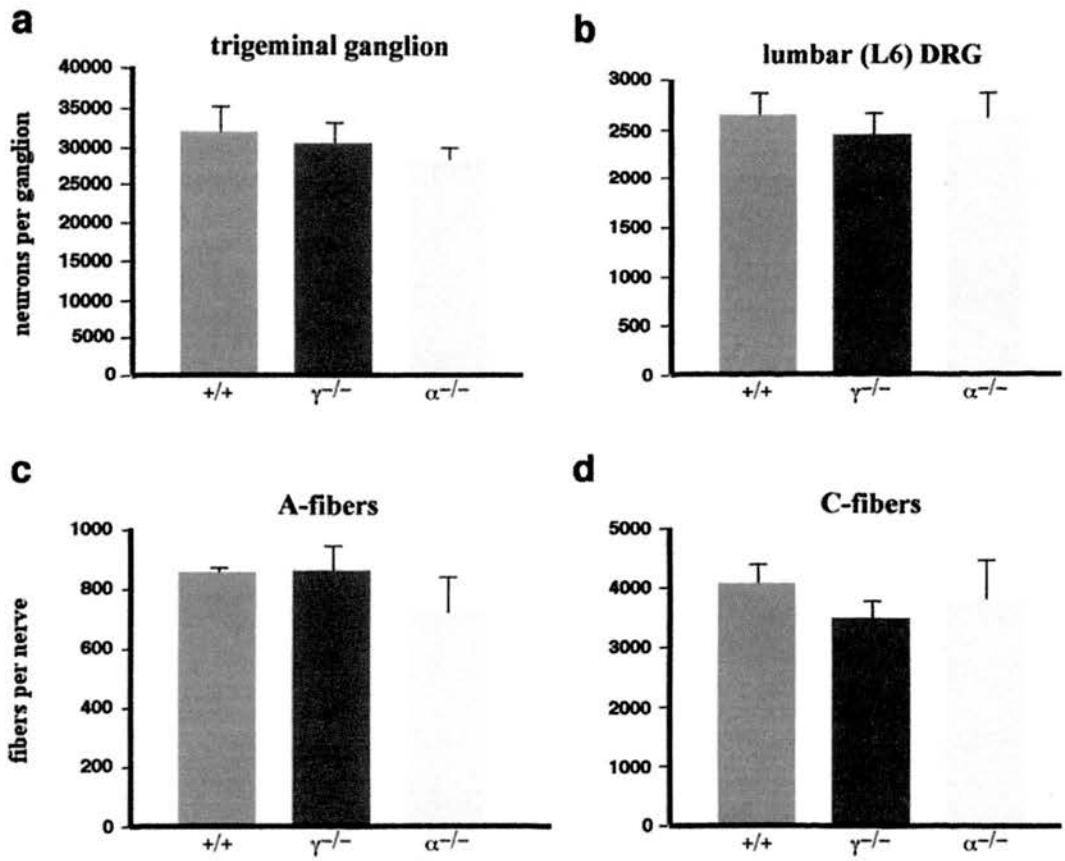

FIG. 6. Numbers of sensory neurons and nerve fibers in wild-type and null mutant mice. (a and b) Average total number of neurons in trigeminal ganglia (a) and L6 lumbar DRG (b) of wild-type (+/+), γ-*synuclein* null mutant ($\gamma^{-/-}$), and α-*synuclein* null mutant ($\alpha^{-/-}$) P2 mice. Means and standard errors of data obtained from analysis of at least 10 ganglia for each genotype are shown. Statistical analysis (Kruskal-Wallis one-way analysis of variance) showed no significant difference in cell numbers between all three genotypes for both ganglia ($P > 0.6$). (c and d) Average total numbers of myelinated A-fibers (c) and unmyelinated C-fibers (d) in adult mouse saphenous nerves. Means and standard errors of data obtained from analysis of at least five nerves for each genotype are shown. Statistical analysis (Kruskal-Wallis one-way analysis of variance) showed no significant difference in numbers for both types of fibers between all three genotypes ($P > 0.4$).

At postnatal day 2 (P2), only neuropil staining (Fig. 3c) or very few neurons with γ-synuclein-positive cytoplasm on the background of neuropil staining (Fig. 3d) are evident in these nuclei. In adult brain all motor nuclei (with the exception of the facial nucleus, where a few neurons still display positive cytoplasmic staining) show a complete absence of γ-synuclein in the cytoplasm of neuronal cell bodies, but axons in all studied motor nerve roots display intense γ-synuclein immunoreactivity (Fig. 4). Using immunofluorescence, we clearly demonstrated that γ-synuclein is present not only in axons but also in presynaptic terminals of motoneurons in neuromuscular junctions (Fig. 4A).

Intracellular localization of γ-synuclein in mouse sensory neurons. As in motoneurons, in sensory neurons of embryonic peripheral ganglia γ-synuclein is distributed throughout the cytoplasm of cell bodies and axons (Fig. 2 and data not shown). However, in contrast to the case for motoneurons, γ-synuclein compartmentalization in sensory neurons does not change during late embryonic and postnatal development. Postnatal neurons of DRG and cranial sensory ganglia display intensive immunostaining of their cell bodies as well as nerve fibers with anti-γ-synuclein antibody (Fig. 3a and b and data not shown).

Numbers of motoneurons in cranial nuclei of wild-type and γ-*synuclein* null mutant mice. Serial coronal sections of adult mouse brain were prepared and stained, and motoneurons were counted in five cranial nuclei as described in Materials and Methods. In neither of these ganglia were statistically significant differences in the number of neurons between wild-type and γ-*synuclein* null mutant mice found (Fig. 5).

Numbers of sensory neurons in DRG and trigeminal ganglia of wild-type and null mutant mice. The number of neurons in L6 lumbar DRG and trigeminal ganglia was assessed in P2 mice, after the period of physiological cell death in these ganglia. Serial longitudinal sections of the lumbar part of the spinal column and transverse sections of the head were stained and neurons were counted as described in Materials and Methods. Figure 6a and b show that the absence of γ-synuclein does not have an effect on the number of neurons in both sensory ganglia. Similarly, no effect of the absence of α-synuclein on the neuronal complement in these ganglia was found. Mice with a targeted inactivation of the α-*synuclein* gene have been described previously (1), but for this work they were further backcrossed with C57BL6J mice (as described in Materials and

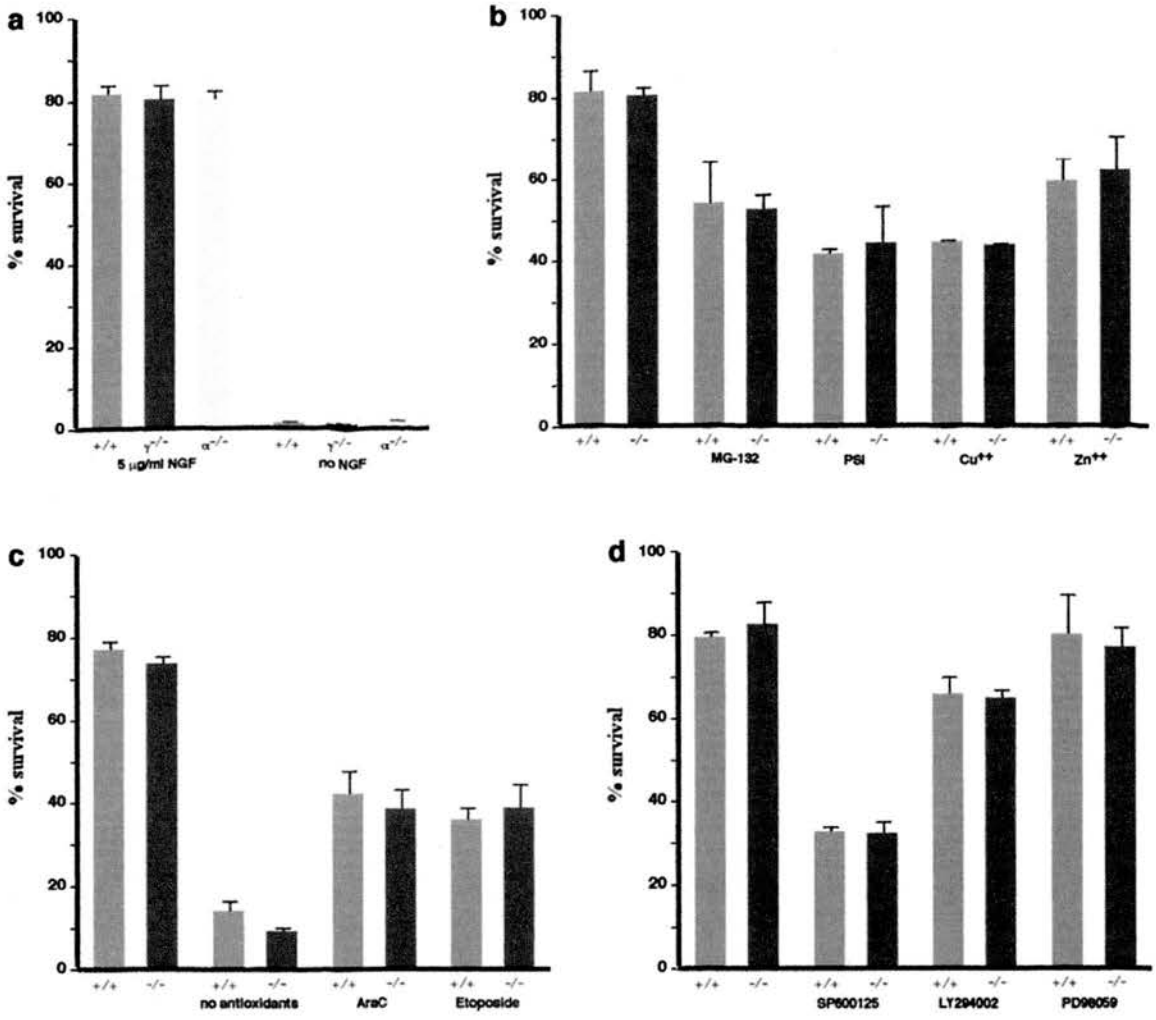

FIG. 7. Survival of P2 mouse trigeminal ganglion neurons in dissociated primary culture. Cultures were prepared and treated with drugs as described in Materials and Methods. Bar charts illustrate survival of neurons 48 h after initial count and addition of drugs. The number of surviving neurons is expressed as a percentage of the initial count. Means and standard errors of data obtained from analysis of at least six culture dishes for each genotype in two independent experiments are shown. (a) Both γ-synuclein-deficient ($\gamma^{-/-}$) and α-synuclein-deficient ($\alpha^{-/-}$) neurons have the same survival rate as wild-type (+/+) neurons in the presence of nerve growth factor (NGF) and are unable to survive in its absence ($P > 0.8$, Kruskal-Wallis one-way ANOVA). (b to d) Various treatments have the same effect on γ synuclein deficient (−/−) neurons as they have on wild-type (+/+) neurons ($P > 0.5$ for all conditions; two-tailed, unpaired Student's t test). Proteasome inhibitors (5 μM MG-132 or 10 μM proteasome inhibitor I [PSI]) or heavy metal ions (30 μM $CuSO_4$ or 75 μM $ZnSO_4$) were added to neurons after the initial count at 24 h after plating (b). In one set of experiments neurons were plated in neurobasal medium supplemented with B27 without antioxidants (c). In other cases, DNA-damaging agents (10 μM cytosine arabinoside [AraC] or 10 μM Etoposide) or inhibitors of the JNK signaling pathway (20 μM SP600125), ERK signaling pathway (20 μM PD98059), or phosphatidylinositol 3-kinase signaling pathway (20 μM LY294002) were added to neurons after the initial count at 3 h after plating (c and d).

Methods) to obtain a colony with a genetic background similar to that of the *γ-synuclein* null mutant mice.

Numbers of nerve fibers in saphenous nerves of wild-type and null mutant mice. To determine whether the absence of synucleins affected growth and myelinization of axons of sensory neurons, we examined the mouse saphenous nerve, which contains mostly afferent sensory nerve fibers, and counted the numbers of myelinated A-fibers and unmyelinated C-fibers. No differences in the general ultrastructural morphology of the fibers (not shown) or in their numbers (Fig. 6c and d) in *γ-synuclein* and *α-synuclein* null mutant mice compared to the wild-type mice were found.

Survival in culture of peripheral nervous system neurons of wild-type and null mutant mice. Although no differences in neuron numbers in DRG or trigeminal ganglia of wild-type, *γ-synuclein*, and *α synuclein* null mutant mice were found, it was feasible to check whether neurons with an incorrect balance of synucleins are more sensitive to various stresses than wild-type neurons. For this we compared the survival of neurons from peripheral ganglia of null mutant and wild type mice in dissociated primary cultures. Dissociated cultures of P2 trigeminal ganglion neurons were prepared and neuronal survival was assessed as described previously (42). Survival characteristics of γ synuclein and α synuclein deficient neurons were

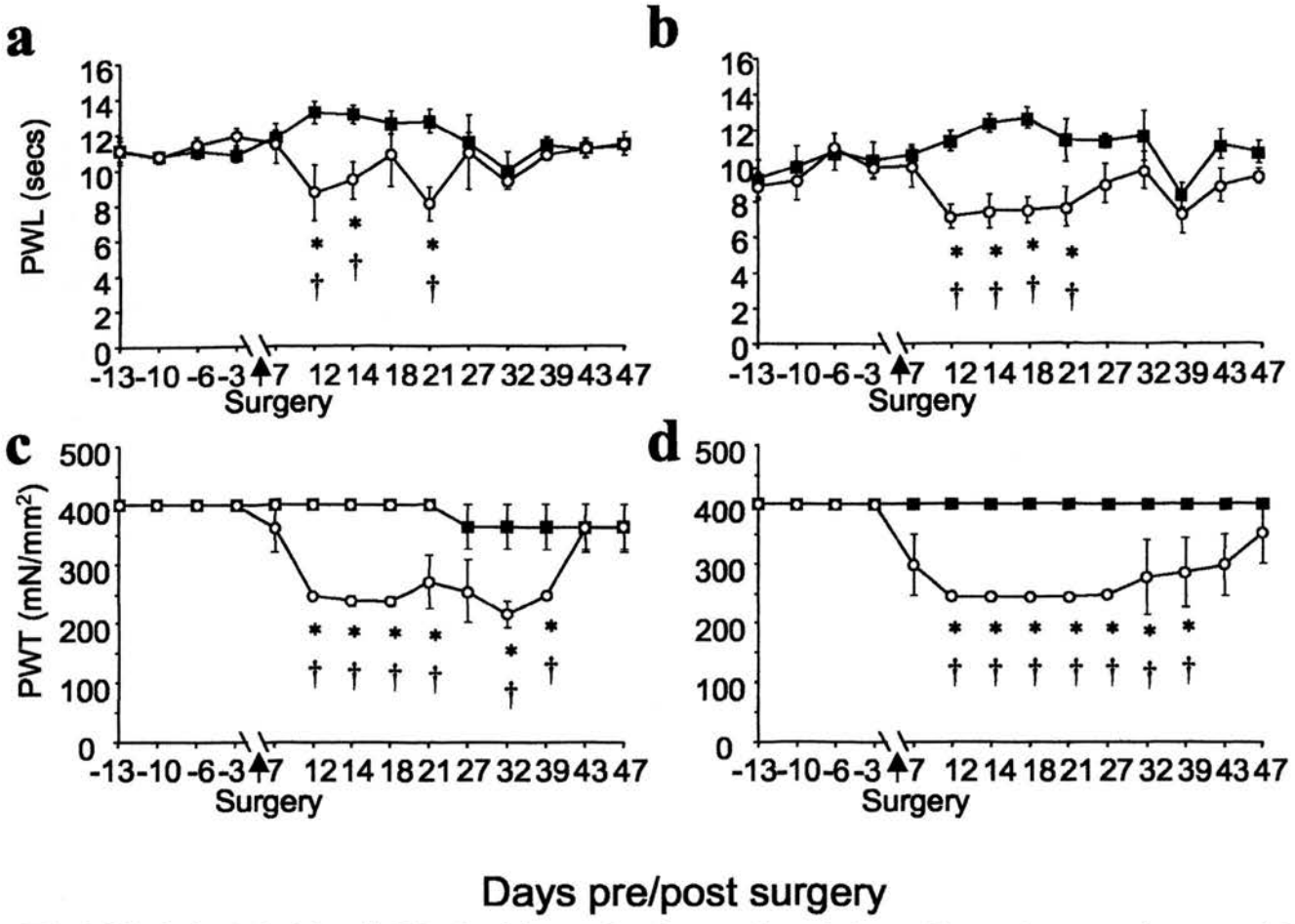

FIG. 8. Behavioral analysis of mice with CCI to the sciatic nerve. Data show mean (± standard error of the mean) responses taken over a period of up to 13 days before and every 2 to 6 days following surgery in wild-type mice (a and c) ($n = 9$) and *γ-synuclein* null mutant mice (b and d) ($n = 6$). (a and b) Paw withdrawal latency (PWL) from a noxious thermal stimulus (Hargreaves' thermal stimulator) ipsilateral to CCI (○) showed significant differences between postoperative and preoperative values (†, $P < 0.05$; Kruskal Wallis one-way analysis of variance) and from postoperative, contralateral (■) values (*, $P < 0.05$ by Student's t test) for both wild-type (a) and mutant (b) mice. No thermal hyperalgesia was seen on the contralateral side. (c and d) Paw withdrawal thresholds (PWT) from mechanical stimulation (von Frey filaments) showed significant differences between postoperative and preoperative values on the side ipsilateral (○) to CCI (†, $P < 0.05$; Dunn's method analysis of variance on ranks) and between postoperative ipsilateral and contralateral (■) values (*, $P < 0.05$, Mann Whitney U test) for both wild type (c) and mutant (d) mice.

indistinguishable from those of wild-type neurons; they survived similarly well in the presence of nerve growth factor and failed to survive in its absence (Fig. 7a). Similar results were obtained for P2 superior cervical ganglion neurons (data not shown). It has been shown previously that certain types of cells with modified expression of synucleins, particularly dopaminergic neurons overexpressing α synuclein, have normal survival characteristics under optimal culture conditions but are substantially more susceptible to stresses (37, 38, 41, 50, 51). To determine whether the absence of γ synuclein renders neurons more sensitive to various toxic insults, we cultivated P2 trigeminal neurons in the absence of antioxidants or in the presence of DNA-damaging agents (Fig. 7c), proteasome inhibitors, heavy metal ions (Fig. 7b), and inhibitors of major intracellular signaling pathways (Fig. 7d) in the medium. None of these treatments revealed differences in the survival of γ-synuclein-deficient neurons compared to wild-type neurons.

Sensory reflexes and effects of CCI in wild-type and *γ-synuclein* null mutant mice. The behavioral reflex responses of adult male *γ-synuclein* null mutant and wild-type littermate mice to noxious radiant heat and graded mechanical stimuli were studied as described in Materials and Methods. In both tests the behaviors of mice from these two groups were indistinguishable (Fig. 8). To examine the effects of sciatic nerve injury on behavioral reflex responses, we used the CCI model of neuropathic pain. Over 7 to 10 days following CCI, wild type and mutant mice progressively developed marked ipsilateral thermal hyperalgesia (reduced paw withdrawal latency) (Fig. 8a and b) and mechanical allodynia (reduced paw withdrawal threshold) (Fig. 8c and d). All responses from the contralateral hind limb remained unaltered. The time courses of recovery from the consequences of CCI were very similar for mice of both genotypes (Fig. 8). These results suggest that degeneration and regeneration of the injured nerve and neuronal plas

ticity in the spinal cord are not compromised in *γ-synuclein* null mutant mice.

DISCUSSION

We have shown that in embryonic motoneurons γ-synuclein is uniformly distributed through the cytoplasm of cell bodies and axons. However, with the exception of a few neurons in the facial nucleus, the cytoplasm of cell bodies of motoneurons of the adult cranial somato- and branchiomotor nuclei is γ-synuclein negative, whereas their axons and synaptic boutons in neuromuscular junctions are intensively stained with anti-γ-synuclein antibody. Previously published in situ hybridization data demonstrated high levels of *γ-synuclein* mRNA in cranial motor nuclei of adult brain (7). Therefore, in motoneurons during postnatal development, γ-synuclein undergoes a compartmentalization shift, which might reflect a functional shift. Our results contradict recently published data (32) which demonstrated the presence of γ-synuclein in cell bodies of motoneurons in brain stem motor nuclei of adult rats. This might reflect a difference between species; however, a more plausible explanation is the difference in the antibodies used in the two studies. In the present study, a highly specific antibody generated against mouse γ-synuclein C-terminal peptide was used, whereas Li et al. (32) used an antibody generated against human recombinant γ-synuclein whose specificity has been checked with recombinant human synucleins but not with samples from null mutant animals.

It is not clear why some neurons of the adult facial nucleus do not follow the common rule and continue to accumulate γ-synuclein in the cytoplasm of their cell bodies. In this aspect they resemble peripheral sensory neurons, in which no developmental changes of intracellular compartmentalization of γ-synuclein take place and which have equally high levels of γ-synuclein in their cell bodies and processes during embryogenesis and postnatally. We found that both neural crest-derived DRG and placode-derived trigeminal sensory neurons have this unchanging pattern of γ-synuclein intracellular compartmentalization. Taken together, our expression studies demonstrated that between neurons expressing the highest levels of γ-synuclein throughout development, two subpopulations could be specified. The first includes peripheral sensory neurons and some motoneurons of the facial nucleus, which localize γ-synuclein in their cell bodies and axons at all developmental stages. Most other motoneurons of the brain stem nuclei comprise the second group, which is characterized by the developmental shift of γ-synuclein compartmentalization.

The high levels of expression suggest that γ-synuclein should have an important role in the development and function of sensory and motoneurons. Consequently, the loss of this protein could affect the morphology and/or physiology of animal sensory and motor systems. To check this, we produced mutant mice with complete inactivation of the *γ-synuclein* gene. Similarly to previously reported *α-synuclein* null mutants (1, 8, 46, 48), these mice showed no obvious phenotypical changes. Detailed studies of sensory and motoneurons in vivo and in vitro failed to detect any difference between *γ-synuclein* null mutant and wild-type mice. The number of neurons is not changed in either of the subpopulations described above, suggesting that proliferation, migration, differentiation, or programmed cell death is not affected by the absence of γ-synuclein. Consistently, survival of γ synuclein deficient neurons in primary culture is not different from survival of wild type neurons. Moreover, the absence of γ synuclein does not render these neurons either more or less sensitive to any of the survival-affecting factors studied so far. Because mid brain dopaminergic neurons seem to be most vulnerable to changes of synuclein metabolism (references 10, 28, and 33 and references therein), it is feasible that this neuronal population might be more sensitive to changes in the synuclein ratio than motoneurons and sensory neurons. We are currently testing whether the absence of γ-synuclein affects survival of dopaminergic neurons in the substantia nigra and ventral tegmental area of null mutant mice.

It has been suggested previously that γ-synuclein could be involved in axonal growth and stabilization of axon architecture (6). However, we did not find differences in the morphology and number of myelinated or unmyelinated fibers in the saphenous nerves of mutant and wild-type mice. Sensory reflex thresholds were also intact in *γ-synuclein* null mutant mice. Nerve injury led to similar changes in sensory function in wild-type and mutant mice. Normalization of sensory function after nerve injury is believed to be associated with neuronal plasticity in the spinal cord and axonal regeneration processes in the injured peripheral nerve (9, 14, 21, 58). These processes require remodeling of the axonal cytoskeleton, including the neurofilament network, and involvement of γ synuclein in regulation of neurofilament network integrity has been suggested previously (6). However, the time course of sensory recovery after CCI in *γ-synuclein* null mutant mice is the same as that in wild-type mice, which suggested that nerve regeneration and plasticity of early somatosensory pathways in *γ-synuclein* mutant mice were unaffected.

The most straightforward explanation of our results is that despite high levels of expression, γ-synuclein is not essential for the development and function of motor and peripheral sensory neurons. Nevertheless an alternative explanation is also possible. This function(s) could be vital for vertebrate organisms, and therefore effective mechanisms of protection against its loss have been developed in evolution. The presence of three closely related synucleins in all vertebrates and substantial overlapping of their expression patterns readily suggest that they are able to compensate for each other's function(s). It is unlikely that compensation for the loss of one synuclein function in mice is achieved by a simple increase of expression of other synucleins. We found no difference in the levels of mRNAs encoding the two remaining synucleins in several neuronal populations of *γ-synuclein* null mutant mice, and the same has been demonstrated before for *α-synuclein* null mutant mice (1). However, it is possible that it is not necessary to boost an already high level of synuclein expression, because changes in compartmentalization, posttranslational modifications, or interaction with other macromolecules could be required and sufficient for functional compensation. Detailed studies of these processes in synuclein null mutant mice as well as studies of double and triple synuclein mutants should shed more light on this problem and finally reveal the normal functions of these proteins.

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Developmental loss and resistance to MPTP toxicity of dopaminergic neurones in substantia nigra pars compacta of γ-synuclein, α-synuclein and double α/γ-synuclein null mutant mice

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Abstract

The growing body of evidence suggests that intermediate products of α-synuclein aggregation cause death of sensitive populations of neurones, particularly dopaminergic neurones, which is a critical event in the development of Parkinson's disease and other synucleinopathies. The role of two other members of the family, β-synuclein and γ-synuclein, in neurodegeneration is less understood. We studied the effect of inactivation of γ-*synuclein* gene on mouse midbrain dopaminergic neurones. Reduced number of dopaminergic neurones was found in substantia nigra pars compacta (SNpc) but not in ventral tegmental area (VTA) of early post-natal and adult γ-synuclein null mutant mice. Similar reductions were revealed in α-synuclein and double α-synuclein/γ-synuclein null mutant animals. However, in none of these mutants did this lead to significant changes of striatal dopamine or dopamine metabolite levels and motor dysfunction. In all three studied types of null mutants, dopaminergic neurones of SNpc were resistant to methyl-phenyl-tetrahydropyridine (MPTP) toxicity. We propose that both synucleins are important for effective survival of SNpc neurones during critical period of development but, in the absence of these proteins, permanent activation of compensatory mechanisms allow many neurones to survive and become resistant to certain toxic insults.

Keywords: development, knockout mice, MPTP, substantia nigra, synuclein.

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Discovery of two point mutations in the human α-*synuclein* gene associated with autosomal dominant familial form of Parkinson's disease (Polymeropoulos *et al.* 1997; Kruger *et al.* 1998) triggered numerous studies aimed at understanding the mechanism of α-synuclein involvement in neurodegeneration. Although various observations suggested a causative role of α-synuclein aggregation in development of synucleinopathies (Spillantini *et al.* 1997, 1998a, 1998b; Conway *et al.* 1998, 2000; Irizarry *et al.* 1998; Mezey *et al.* 1998; Wakabayashi *et al.* 1998; Arai *et al.* 1999; Lippa *et al.* 1999; Narhi *et al.* 1999; Serpell *et al.* 2000; Takeda *et al.* 2000; Mori *et al.* 2002), it has also been shown that α-synuclein could cause cell death or render cells more sensitive to toxic insults independently of protein fibrillation (Ostrerova *et al.* 1999; Hsu *et al.* 2000; Saha *et al.* 2000; Lee *et al.* 2001; Gosavi *et al.* 2002; Lehmensiek *et al.* 2002; Petrucelli *et al.* 2002). This apparent discrepancy was at least partially explained when a multistep mechanism of α-synuclein aggregation was revealed and the cytotoxicity was attributed to soluble α-synuclein oligomers or protofibrils, rather than insoluble highly polymerized mature fibrils (Wood *et al.* 1999; Stefanis *et al.* 2001; Volles *et al.* 2001; Ding *et al.* 2002; Gosavi *et al.* 2002; Lashuel *et al.* 2002; Volles and Lansbury 2002; Zhu *et al.* 2003).

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Abbreviations used: DA, dopamine; DAB, diaminobenzidine; DOPAC, 3,4-dihydroxyphenylacetic acid; FITC, fluorescein isothiocyanate; 5-HIAA, 5-hydroxyindolacetic acid; HPLC, high-performance liquid chromatography; HVA, homovanillic acid; MPTP, methyl-phenyl-tetrahydropyridine; NCD, natural cell death; PBS, phosphate-buffered saline; SDS–PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; SNpc, substantia nigra pars compacta; SNpc, substantia nigra pars compacta; TH, tyrosine hydroxylase; VTA, ventral tegmental area.

Overexpression of α-synuclein does not always have detrimental effects on the survival of cells in culture and, in some cells, has been shown to protect them from certain insults (Alves Da Costa *et al.* 2002; Lotharius *et al.* 2002; Wersinger and Sidhu 2003; Zourlidou *et al.* 2003). This suggests that the increase of intracellular concentration of α-synuclein is not the sole factor that changes cell fate; the presence and activity of certain macromolecules and/or intracellular structures and pathways, which interact either physically or functionally with α-synuclein are of no less importance. Among the factors shown to affect cell survival reciprocally with α-synuclein are free radical-producing metabolic pathways, excitotoxicity, signal transduction pathways, molecular chaperons, intracellular trafficking machinery, mitochondrial dysfunction and protein degradation systems, primarily proteasome (for recent reviews see Betarbet *et al.* 2002; Bonini 2002; Hardy 2002; Helfand 2002; Kruger *et al.* 2002; Lotharius and Brundin 2002; Mattson *et al.* 2002; Schwartz *et al.* 2002; Welch and Yuan 2002; Wolozin and Golts 2002; Cookson 2003; Dev *et al.* 2003; Lee 2003; Volles and Lansbury 2003).

Recently, attention was focussed on the possible role of two other members of the family, β-synuclein and γ-synuclein, in the regulation of α-synuclein-induced neurotoxicity. Although these two proteins are similar to α-synuclein in their amino acid sequences, they display rather different aggregation kinetics and do not form amyloid-like fibrils (reviewed in Uversky and Fink 2002). No mutations/polymorphisms have been found so far in *β-synuclein* or *γ-synuclein* genes in association with any neurodegenerative diseases (Lavedan *et al.* 1998b; Flowers *et al.* 1999; Lincoln *et al.* 1999a, 1999b; Kruger *et al.* 2001). Likewise, these two proteins have never been detected in histopathological hallmarks of neurodegeneration, although their presence in unusual structures or changes of their intracellular distribution have been reported in several cases of human neurological diseases (Duda *et al.* 1999; Galvin *et al.* 1999, 2000; Mori *et al.* 2002). Most interestingly, β-synuclein and γ-synuclein inhibit the generation of α-synuclein protofibrils and fibrils *in vitro* (Uversky *et al.* 2002; Windisch *et al.* 2002; Park and Lansbury 2003), and are able to prevent neurotoxic effects of α-synuclein at least in certain *in vivo* systems (Hashimoto *et al.* 2001; Windisch *et al.* 2002; da Costa *et al.* 2003; and our unpublished observations).

Taken together these suggest that correct balance of synucleins is important for normal brain function and imbalance of these proteins might affect survival of neurones that normally express more than one synuclein. However, recently we have shown that targeted inactivation of *γ-synuclein* gene in mice does not affect survival of peripheral sensory and motor neurones that express high levels of this protein in wild-type animals (Ninkina *et al.* 2003). Nevertheless, because of compelling evidence that midbrain dopaminergic neurones are more vulnerable to changes of synuclein metabolism than other neuronal populations (Zhou *et al.* 2000, 2002; Xu *et al.* 2002) it was expedient to study the effects of *γ-synuclein* gene inactivation on these neurones.

Here we demonstrate that in mouse SNpc, deficiency of γ-synuclein, α-synuclein or both of these proteins, has negative effect on development of dopaminergic neurones, but renders them resistant to the toxic effect of MPTP.

Experimental procedures

Materials

Methyl-phenyl-tetrahydropyridine (MPTP), diaminobenzidine (DAB; Sigma Fast 3,3′-diaminobenzidine tablet sets) and general chemicals were purchased from Sigma (St Louis, MO, USA), Vectastain ABC-kit from Vector Laboratories (Peterborough, UK), fluorescein isothiocyanate (FITC)-conjugated anti-mouse and TRITC-conjugated anti-rabbit immunoglobulins from Jackson ImmunoResearch Laboratories (West Grove, PA, USA).

Generation of double γ-synuclein/α-synuclein null-mutant mice

Targeting inactivation of *γ-synuclein* gene in ES cells and production of null mutant mice on pure C57Bl6J (Charles River Laboratories, Wilmington, MA, USA) background were described previously (Ninkina *et al.* 2003). A colony of α-synuclein mutant mice on pure genetic background was established from mice described previously (Abeliovich *et al.* 2000) by backcrossing with C57Bl6J mice (Charles River) for several generations as described elsewhere (Ninkina *et al.* 2003). Null mutant α-synuclein and γ-synuclein mice were bred to produce double heterozygous and consequently double γ-synuclein/α-synuclein null mutant mice.

Immunoblotting

Substantia nigra and surrounding midbrain region were dissected from adult mouse brains and homogenised in sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) loading buffer followed by incubation of lysates for 5 min at 100°C. To avoid the influence of individual differences and inconsistent dissections each lysate was prepared from tissues dissected from four brains. Samples of these lysates (10 μg of total protein) were analysed in 18% SDS–PAGE, transferred to Hybond-P membrane (Amersham, Arlington Heights, IL, USA) and probed with antibody as described previously (Buchman *et al.* 1998; Ninkina *et al.* 1999). Affinity-purified polyclonal rabbit SK23 antibody generated against C-terminal peptide of mouse γ-synuclein (persyn; Buchman *et al.* 1998) was used in 1 : 500 dilution, sheep polyclonal antiα-synuclein antibody (AB5334P, Chemicon, Temecula, CA, USA) – in 1 : 1000 dilution, rabbit polyclonal antiβ-synuclein antibody (AB5086, Chemicon) – in 1 : 3000 dilution and mouse monoclonal antiα-tubulin antibody (DM1A, Sigma) – in 1 : 10000 dilution.

Histology

For the quantification of dopaminergic neurones in the midbrain of wild-type and mutant animals, brains were collected from E18 embryos, post natal day 5 (P5) or adult mice. To minimize variations between the specimens, cohorts of brains from animals of the same age group were collected, processed, embedded and stained in parallel. Animals were killed by an appropriate Schedule 1 method

according to the UK Animals (Scientific Procedures) Act 1986. The brains were fixed in 4% paraformaldehyde/PBS (phosphate-buffered saline) or Carnoy's fixative (60% ethanol, 30% chloroform, 10% glacial acetic acid) at 4°C overnight following dehydration in alcohol series and embedding in paraffin blocks. Then, 8-μm-thick sections were cut using a HM 310 microtome (Microm International, Walldorf, Germany) and mounted onto Gold Seal slides (Gold Seal Products, Portsmouth, NH, USA). The paraffin sections were cleared in xylene and rehydrated through a graded alcohol series. Endogenous peroxidase activity was quenched by incubating the slides in 3% H_2O_2 in methanol for 20 min. After washing with PBS the tissues were blocked in 10% horse or goat serum and 0.4% Triton X-100 in PBS for 1 h at room temperature. Incubation with primary antibody was carried out at 4°C overnight. A monoclonal antibody (clone 1B5) against tyrosine hydroxylase was purchased from Novocastra Laboratories (Newcastle upon Tyne, UK) and used in 1 : 40 dilution. Detection of immune complexes with biotinylated anti-mouse or anti-rabbit antibodies and avidin/peroxidase complex from Vectastain ABC-kit, and DAB as substrate were carried out according to manufacturers instructions. For analysis of γ-synuclein protein expression and intracellular localization sections of the whole E15 embryos and E18, P2 or adult brains were prepared as described above and probed with 1 : 40 dilution of an SK23 antibody. For double immunoflourescent studies, FITC-conjugated anti-mouse and TRITC-conjugated anti-rabbit immunoglobulins were used in 1 : 200 dilution.

Cell counts

To assess number of dopaminergic neurones in SNpc and VTA regions of mouse brain, the first section for counting was randomly chosen from the first 10 sections that included SNpc/VTA region. Starting from this section, nuclei of tyrosine hydroxylase (TH)-positive cells were counted on the every tenth section through the whole region. The Axiovision imaging program (Carl Zeiss Vision, Munchen-Hallbergmoss, Germany) was employed to measure diameters of nuclei of 50 randomly picked dopaminergic neurones in the SNpc or ventral ventral tegemental area (VTA) of every mouse brain included in this study. The average nuclear diameter for each structure in each brain was used for Abercrombie's correction (Abercrombie 1946) of nuclei counts to obtain an actual number of TH positive cells in the structure. All counts were carried out blindly by a person unaware of the genotype of the animals.

MPTP treatment

Studies were approved by the Home Office and carried out according to the UK Animals (Scientific Procedures) Act 1986. All procedures, which included MPTP handling were carried out in accordance with published safety recommendations (Przedborski *et al.* 2001a). Eight- to 10-week-old wild-type or mutant male mice on C57Bl6 background were injected intrperitoneally (i.p.) with 0.1 mL of PBS or MPTP dissolved in PBS at 24-h intervals for 5 days. Daily dose of MPTP was 30 mg/kg. Brains were collected for histological studies 21 days after the last injection.

Rotarod testing

Seven-month-old male animals were tested thrice on a rotarod (UGO Basil, Comerio, Italy) at constant (24 rpm) rotation speed for 180 s or in accelerating (from 4 to 40 rpm) mode for 300 s. Results were expressed as mean ± SEM of seconds before fall for each experimental group.

Measurement of striatal dopamine and dopamine metabolite levels

Brains of 9-month-old wild-type or mutant male mice on C57Bl6 background were dissected, the striatum removed on ice, snap-frozen and kept at −70°C until assayed. 95 μL of 0.4 M $HClO_4$ and 5 μL of 40 μg/mL N-ω-5-HT (internal standard) was added to each thawed sample prior to sonication for 3 s by an ultrasonics homogenizer with a 3 mm tip. The samples were centrifuged at 20 000 *g* at a benchtop centrifuge for 25 min at 4°C. The pellet was frozen for protein quantification, which was carried out using a standard BCA protein assay reagent kit (Pierce, Rockford, IL, USA). Then, 50 μL of the supernatant were injected onto the high-performance liquid chromatography (HPLC) column through a Rheodyne injection valve connected to a 20 μL loop. A BAS PM-80 solvent delivery system and BAS LC-4 ECD was used to detect dopamine (DA) and its metabolites (DOPAC, HVA and 5-HIAA). Isocratic mobile phase (75 mM sodium dihydrogen phosphate, 1.7 mM octanosulphonic acid sodium salt, 100 μL/L triethylamine in 90% milliQ water, 10% acetonitrile, pH 3.0) was used to carry the samples through a reverse phase ESA column (120 A C18 150 × 3.2 mm column packed with 3-μm particles). Flow rate was set at 0.6 mL/min and the detector was at 0.7 V.

Results

Intracellular compartmentalisation of γ-synuclein in substantia nigra neurones changes during development

SK23 antibody, which specifically recognizes mouse γ-synuclein was used to stain paraffin sections of embryonic and post-natal mouse brains. In agreement with previously published *in situ* hybridization data, expression of γ-synuclein in neurones of subsatantia nigra or its primodium was detected at all developmental stages studied, although intracellular compartmentalisation of the protein was different at different stages. At E15, γ-synuclein was detected in the primodium of substantia nigra both in neuronal cell bodies and in axons (Figs 1a and b). Later in embryonic and post-natal development, γ-synuclein disappears from perikaria (Figs 1d and e), whereas axons of the nigro-striatal tract remain intensively stained (Figs 1c and g). However, in adult SNpc, haphazard TH-positive neurones display positive γ-synuclein immunostaining in their cell bodies (Figs 1i and j). In developing and adult brain, punctuate neuropil staining was detected in striatum (data not shown), suggesting that γ-synuclein is localized not only in axons but also in pre-synaptic regions of dopaminergic neurones of SNpc. Thus, neurones of the mouse nigro-striatal system display the same developmental dynamics of intracellular compartmentalization of γ-synuclein as brain stem motoneurones (Nink ina *et al.* 2003), another neuronal population expressing high levels of this protein. Recently, we generated γ-synuclein

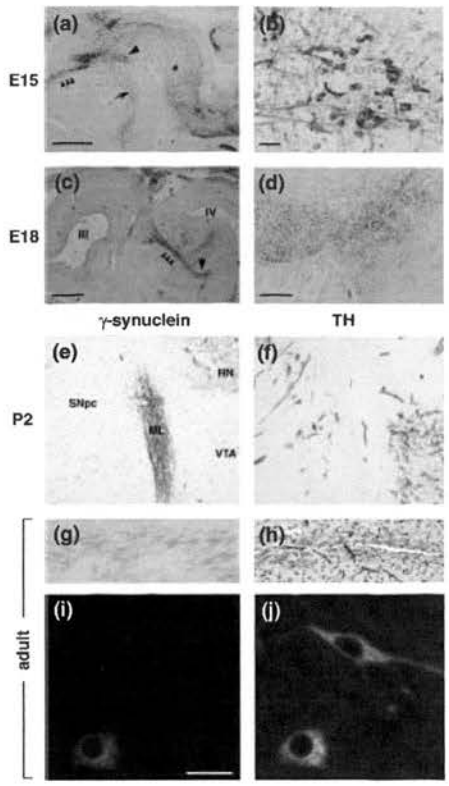

Fig. 1 Intracellular comparetmentalisation of γ-synuclein in developing and adult mouse nigro-striatal system. Immunostaining of paraffin sections of mouse E15 (a, b), E18 (c, d), P2 (e) or adult (g) brains with anti-γ-synuclein antibody. In a sagittal section of E15 mouse brain (a), three small arrowheads denote the strongly stained fasciculus retroflexus, an important landmark in identifying the primordium of the substantia nigra (large arrowhead). An arrow designates the positively stained nigro-striatal tract. At a higher magnification (b), labelled cell bodies of the primordium of substantia nigra are visible. In a sagittal section of E18 mouse brain (c), the third and fourth ventricles are shown; other designations as in (a). At the higher magnification (d), no labelled cell bodies are visible but the primordial region is clearly outlined by a dotted neuropil stain. In a coronal section of P2 mouse brain (e), cell bodies of neurones in the red nucleus (RN) and nerve fibres in medial lemniscus (ML) are stained but no γ-synuclein-positive cell bodies are apparent in VTA or SNpc. An adjacent section stained with anti-TH antibody to delineate VTA and SNpc is shown in (f). A fragment of sagittal section of adult mouse brain shows γ-synuclein-positive axons in the nigro-striatal tract (g); an adjacent section stained with anti-TH antibody and counterstained with H&E (h). Double-immunoflourescent staining of adult SNpc neurones with anti-γ-synuclein (i) and anti-TH (j) antibody. Scale bars are 500 μm (a and c), 20 μm (b) and 50 μm (d).

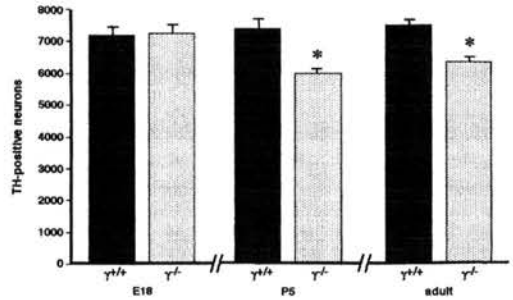

Fig. 2 The number of dopaminergic neurones in midbrain of wild-type and γ-synuclein null mutant mice. Bar chart shows mean ± SEM of total number of TH-positive neurones in SN + VTA of E18, P5 and adult mice. No difference was found in E18 ($p > 0.5$, Student's *t*-test; $n = 6$ for each genotype) but P5 and adult γ-synuclein null mutant mice ($n = 8$ and 6, respectively) have significantly less ($^*p < 0.05$, Student's *t*-test) neurones than adult wild-type mice ($n = 9$).

null mutant mice and demonstrated that the number of motoneurones in several brain stem nuclei is not affected by targeted inactivation of γ-*synuclein* gene (Ninkina *et al.* 2003). However, various data suggest that dopaminergic neurones might be more susceptible to changes in metabolism of synucleins than other types of neurones (Zhou *et al.* 2000, 2002; Kirik *et al.* 2002; Xu *et al.* 2002). Therefore, we studied the number of dopaminergic neurones in midbrain structures of γ-synuclein null mutant mice.

Deficit of dopaminergic neurones in substantia nigra pars compacta of γ-synuclein null mutant mice

In embryonic mouse brain, topographical discrimination between dopaminergic neurones of SN and VTA is problematic. Therefore, we compared the total (SN + VTA) numbers of TH-positive neurones in midbrain of wild-type and γ-synuclein null mutant E18 embryos. At this developmental stage the same number of dopaminergic neurones was observed in SN + VTA of γ-synuclein null and wild-type mice (Fig. 2). However, in early post-natal (P5) and adult (15–20 weeks) animals, small (15–20%) but statistically significant deficit of TH-positive neurones in SN + VTA of γ-synuclein null mutant has been found (Fig. 2). To reveal if both structures lost the same proportion of neurones during post-natal development we studied more samples and counted separately the number of TH-positive neurones in SNpc and VTA of these two groups of adult mice. The results shown in Fig. 3 clearly demonstrate that the loss of dopaminergic neurones in γ-synuclein null mutant mice takes place in SNpc (85.5 ± 5.2% of the number of neurones in wild-type mice) but not in VTA (102.5 ± 5.0% of the number of neurones in wild-type mice). In 18-month-old animals, the oldest studied so far, we found similar difference in the number of TH-positive neurones in SNpc between γ-synuclein null mutant mice and wild-type littermates (81.4 ± 3.5% of the number of neurones in wild-type mice).

Double γ-synuclein/α-synuclein null mutant mice

Inactivation of γ-*synuclein* gene ultimately results in an increase of α-synuclein to γ-synuclein ratio in cells that

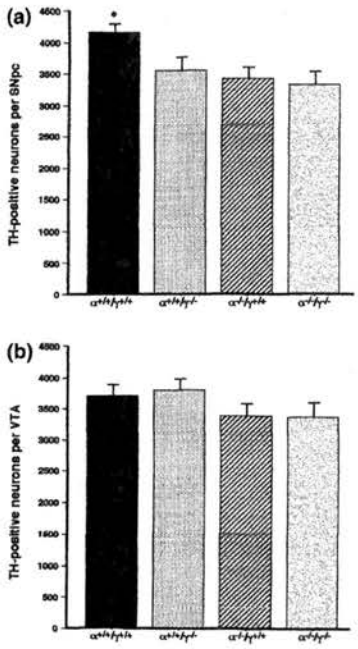

Fig. 3 The number of dopaminergic neurones in midbrain structures of wild-type and synuclein null mutant mice. Bar charts show mean ± SEM of total number of TH-positive neurones in SNpc (a) or VTA (b) of wild-type ($\alpha^{+/+}/\gamma^{+/+}$), γ-synuclein null mutant ($\alpha^{+/+}/\gamma^{-/-}$), α-synuclein null mutant ($\alpha^{-/-}/\gamma^{+/+}$) and double null mutant ($\alpha^{-/-}/\gamma^{-/-}$) mice. Neurones were counted separately in left and right structures of at least seven animals for each genotype. Statistic analysis (Kruskal–Wallis one-way ANOVA) showed no difference in VTA ($p > 0.3$) but significantly reduced number of neurones in SNpc ($p < 0.01$) of all three types of mutant mice when compared to wild-type mice.

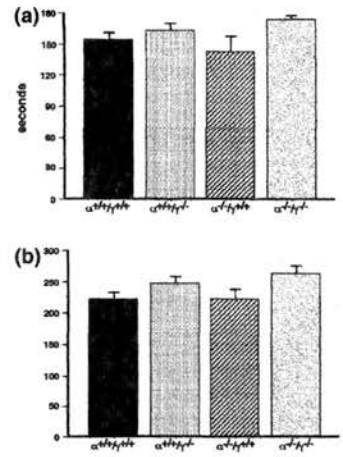

Fig. 4 Performance of wild-type and synuclein null mutant mice in rotarod tests. Bar charts show mean ± SEM of time intervals from the test start to animal fall from the rotating rod. Results of 3 min test with constant, 24 rpm, rotation speed (a) and 5 min accelerating rotation test (b) are shown. Statistic analysis (Kruskal–Wallis one-way ANOVA) showed no significant difference in performance of wild-type and mutant mice in both tests ($p > 0.4$).

normally express these two proteins, including dopaminergic neurones of SNpc. As such imbalance might be responsible for the increased toxicity of α-synuclein in the most vulnerable neuronal population, we produced mice lacking both α-synuclein as well as γ-synuclein and studied neuronal complement in their SNpc and VTA.

For these studies, α-synuclein-deficient mice on the pure C57Bl6J genetic background (Ninkina *et al.* 2003) were first crossed with γ-synuclein-deficient mice on the same pure genetic background, to produce double heterozygous animals. Mice lacking both *α-synuclein* and *γ-synuclein* genes were obtained in litters from double heterozygous parents with expected Mendelian frequency. Similar to both single null mutants, double null mutant mice were viable, fertile and did not show any obvious abnormalities in development, behaviour or in gross morphology of the nervous system (data not shown). Neither of our three populations of mutant mice developed motor dysfunction, as can be judged from their performance in either constant speed (Fig. 4a) or accelerated (Fig. 4b) rotarod tests.

The number of TH-positive neurones in SNpc and VTA of adult double α-synuclein/γ-synuclein null mutant mice was similar (79.9 ± 5.3% and 91.4 ± 5.9% of the number of neurones in wild-type mice, respectively) to the number of TH-positive neurones in the same structures of γ-synuclein null mutant mice (Fig. 3). Moreover, a similar deficit in the number of TH-positive neurones was found in SNpc (82.2 ± 4.4% of the number of neurones in wild-type mice) but not VTA (91.6 ± 5.3% of the number of neurones in wild-type mice) of α-synuclein null mutant mice (Fig. 3).

To assess the effect of null mutation on the levels of dopamine and its metabolites, striatum was dissected from nine months old male mice and HPLC analysis was used to measure DA, DOPAC, 5-HIAA and HVA levels in the extract of each of individual striatum. For all four neurochemicals, no statistically significant differences were found between four studied genotypes (Fig. 5).

Dopaminergic neurones of single and double synuclein deficient mice are resistant to MPTP toxicity

To address the question of whether the absence of synucleins changes the sensitivity of dopaminergic neurones to specific toxic insults, we treated α-synuclein, γ-synuclein and double α-synuclein/γ-synuclein null mutant mice with MPTP, a neurotoxic drug that affects predominantly dopaminergic neurones of SNpc. A protocol of MPTP administration (see Experimental procedures) resulting in apoptotic death of dopaminergic neurones (Tatton and Kish 1997) has been chosen. This protocol allows for the identification both of animals more sensitive as well as less sensitive to the neurotoxic effect of the drug because it

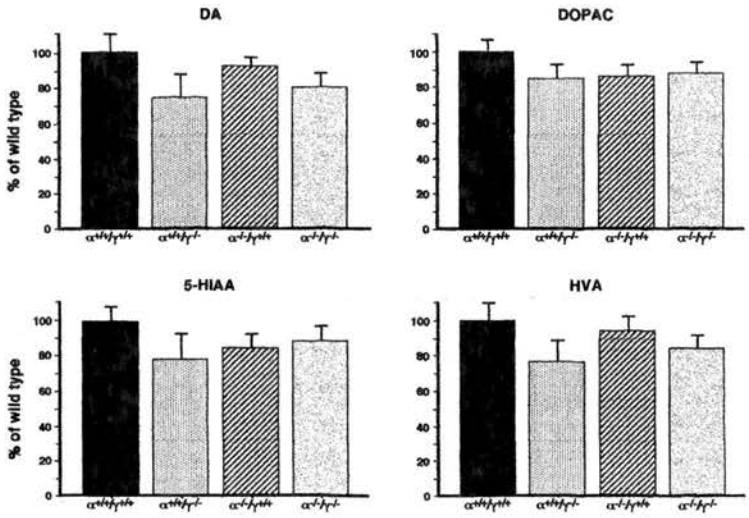

Fig. 5 Dopamine and its metabolite levels in striatum of wild-type and synuclein null mutant mice. Striatal concentrations (ng/mg protein) of dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), 5-hydroxyindolacetic acid (5-HIAA) and homovanillic acid (HVA) in mutant animals were normalized to corresponding mean values for wild-type animals (100%) in each experiment. Mean ± SEM for nine animals per genotype from two separate experiments are shown. Statistical analysis showed no significant difference between wild-type and mutant mice for all four neurochemicals ($p > 0.1$, one-way ANOVA with posthoc Newman–Keuls test).

causes only moderate reduction in the number of TH-positive neurones in SNpc of treated wild-type mice, which is not accompanied by substantial decrease of striatal dopamine level and, consequently, do not compromise animal performance in rotarod tests. As expected, in a group of wild-type mice this treatment substantially reduced the number of TH-positive neurones in SNpc (64.7 ± 11.1% of neurones remain in MPTP-treated group; 100% is the mean of neurone numbers in a vesicle-treated group of wild-type animals, $p < 0.05$) without significant effect on rotarod performance of these mice (Fig. 6).

However, no significant decrease in number of TH-positive neurones after MPTP treatment was observed in SNpc of mice lacking either α-synuclein (88.1 ± 4.7% of neurones remain in MPTP-treated group; 100% is the mean of neurone numbers in a vesicle-treated group of α-synuclein deficient animals, $p > 0.1$), or γ-synuclein (95.3 ± 7.4% of neurones remain in MPTP-treated group; 100% is the mean of neurone numbers in a vesicle-treated group of γ-synuclein deficient animals, $p > 0.6$), or both synucleins (85.9 ± 6.6% of neurones remain in MPTP-treated group; 100% is the mean of neurone numbers in a vesicle-treated group of double null mutant animals, $p > 0.2$; Fig. 6).

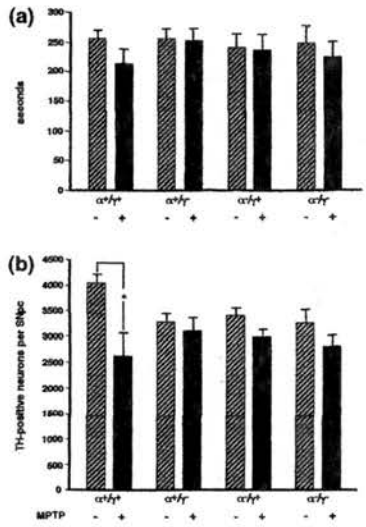

Fig. 6 Effect of chronic MPTP treatment on wild-type and synuclein null mutant mice. (a) Performance of wild-type (α^+/γ^+), γ-synuclein null mutant (α^+/γ^-), α-synuclein null mutant (α^-/γ^+) and double null mutant (α^-/γ^-) mice in 5 min accelerating rotarod test before (–) and 2 weeks after (+) MPTP treatment. (b) The number of dopaminergic neurones in SNpc of wild-type and synuclein null mutant treated with MPTP (+) or vehicle (–). Neurones were counted separately in left and right SNpc of at least six animals for each experimental group. Statistic analysis (Kruskal–Wallis one-way ANOVA and Student's *t*-test separately for each genotype) showed significant reduction in the number of neurones after MPTP treatment only for wild-type animals (*, see Results for details).

Increased levels of β-synuclein in midbrain of single and double synuclein deficient mice

Immunoblotting was used to compare levels of synucleins in midbrain of adult wild-type and synuclein null mutant mice. Equal amounts of total protein from four combined midbrains per each genotype were analysed using antibodies specific to α-, β- and γ-synucleins and α-tubulin as a loading control. As expected, α-synuclein was absent in samples of α-synuclein null mutant and γ-synucleins – in samples of γ-synucleins null mutant animals, both these synucleins were absent in samples of double null mutant animals (Fig. 7). In three independent experiments we consistently detected higher levels of β-synuclein in midbrain samples of all three null mutant mouse lines than in samples of wild-type mice (Fig. 7).

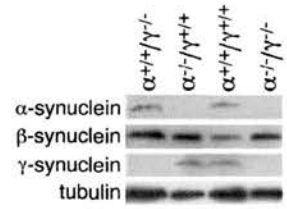

Fig. 7 Expression of synucleins in midbrain of adult mice. Each of three identical western blots with 10 μg of total protein lysates per lane were probed with antibody against α-synuclein, β-synuclein or γ-synuclein. The filter probed with antiβ-synuclein antibody was re-probed with antiα-tubulin antibody (lower panel). The lysates were prepared by homogenisation and boiling in SDS–PAGE loading buffer of midbrains of wild-type ($\alpha^{+/+}/\gamma^{+/+}$), γ-synuclein ($\alpha^{+/+}/\gamma^{-/-}$), α-synuclein ($\alpha^{-/-}/\gamma^{+/+}$) or double null mutant ($\alpha^{-/-}/\gamma^{-/-}$) mice (four combined midbrains for each genotype).

Discussion

In a search for phenotypical manifestations of targeted inactivation of γ-*synuclein* gene in mice we carried out quantitative studies of mesencephalic dopaminergic neurones of SNpc and VTA, neuronal populations, which in wild-type adult mouse express high levels of γ-*synuclein* mRNA (Lavedan *et al.* 1998a; Abeliovich *et al.* 2000; and our unpublished observations). A relatively small (~15%) but consistent and statistically significant reduction in the number of TH-positive neurones has been found in SNpc of adult γ-synuclein null mutant mice when compared with their wild-type littermates. This neuronal loss is not progressive, as the same difference in the number of neurones has been found between groups of ageing mutant and wild-type animals. Reduced number of midbrain dopaminergic neurones is already evident in P5 animals but earlier in development, at the late embryonic stage (E18), γ-synuclein null mutant and wild-type mice have the same number of these neurones, suggesting that observed neuronal loss occurs peri- or early post-natally. It is well established that in rodents the natural cell death (NCD) of dopaminergic neurones in SNpc takes place during this perinatal and early post-natal period (reviewed in Burke 2003). Therefore it is feasible that the absence of γ-synuclein makes dopaminergic neurones more vulnerable to NCD during this critical stage of their development.

Despite the observed neuronal deficit, null mutant mice did not develop any detectable motor dysfunction. This is not surprising because clinical signs of nigro-striatal pathology become obvious only following a substantial decrease of striatal dopamine level, which in turn is usually associated with much more extensive loss of nigral neurones. Indeed, direct measurement of striatal dopamine and its metabolites has not shown significant difference in their levels between wild-type and null mutant animals. In contrast to previously reported data (Abeliovich *et al.* 2000), we have not found reduction of striatal dopamine level in α-synuclein null mutant mice. The most probable explanation of this discrepancy is different genetic background of mice used in two studies. Abeliovich *et al.* (2000) used 129SV/j × C57Bl6 F2 intercrosses and all our experimental animals were transferred on pure C57Bl6 background as described earlier (Ninkina *et al.* 2003). Notably, two other groups that independently produced α-synuclein null mutant mice also did not find statistically significant decrease of their striatal dopamine levels (Cabin *et al.* 2002; Schluter *et al.* 2003).

In the absence of a clear understanding of normal synuclein function, various scenarios can be employed to explain why γ-synuclein null mutant mice develop a neuronal deficit in SNpc and why only a fraction of dopaminergic neurones are lost. Recently, the importance of a correct balance of synucleins for the survival of certain types of neurones has been proposed. Selective neurotoxicity of α-synuclein to dopaminergic neurones has been demonstrated in cell cultures (Zhou *et al.* 2000, 2002; Xu *et al.* 2002) as well as in invertebrate (Feany and Bender 2000; Auluck *et al.* 2002; Lakso *et al.* 2003), rodent (Kirik *et al.* 2002; Lo Bianco *et al.* 2002) and primate (Kirik *et al.* 2003) *in vivo* models. The ability of β-synuclein and γ-synuclein to block formation of toxic intermediates of α-synuclein aggregation (Uversky *et al.* 2002; Windisch *et al.* 2002; Park and Lansbury 2003) and α-synuclein-induced cell death (Hashimoto *et al.* 2001; Windisch *et al.* 2002; our unpublished data) suggested that in the absence of endogenous β-synuclein or γ-synuclein, dopaminergic neurones might become more susceptible to various internal and/or external insults due to the lack of natural counterbalance to the intrinsic toxicity of endogenous α-synuclein. However, our finding that dopaminergic neurones of VTA, which in wild-type mice express both α- and γ-synuclein, are not affected in γ-synuclein null mutant mice, suggests that neuronal loss seen in SNpc could not be explained only by selective toxicity of endogenous α-synuclein for dopaminergic neurones. Also, intracellular α-synuclein-positive inclusions, obvious indicators of the increased propensity of endogenous α-synuclein to aggregate in the absence of γ-synuclein, have never been observed in brain sections of adult and ageing γ-synuclein null mutant mice. Most importantly, we have found the same reduction in the number of TH-positive neurones in SNpc of double α-synuclein/γ-synuclein null mutant mice as in γ-synuclein null mutants. This suggests that the absence of α-synuclein does not rescue a susceptible population of γ-synuclein-negative dopaminergic neurones from developmental cell death. Moreover, we have found similar neuronal deficit in SNpc of α-synuclein null mutant mice. The latter result is consistent with findings of another group, although authors did not comment on the significance of the difference they have shown (Dauer *et al.* 2002).

We believe that the most plausible interpretation of our experimental data is that both α- and γ-synuclein are involved in and equally important for a certain intracellular process required for effective survival of SNpc dopaminergic neurones during a critical period of SNpc development. The roles of α- and γ-synuclein in this process might be complementary, but not interchangeable. Therefore, the same neuronal population is affected in α-synuclein and γ-synuclein null mutant mice and the absence of both these synucleins does not have an additive effect on neuronal survival. Experimental data obtained in various model systems implicate synucleins in many intracellular pathways associated with regulation of cell survival. The next obvious challenge is to reveal which of these pathways are indeed involved in synuclein-dependent survival of dopaminergic neurones of SNpc in the developing brain.

However, it is obvious from our previous (Ninkina *et al.* 2003 and unpublished data) and present data, that in most neurones that normally express high levels of both α- and γ-synuclein, including the majority of dopaminergic neurones of SNpc, the absence of these proteins are compensated and this allows them to survive through all critical stages of development. Furthermore, synuclein-deficient dopaminergic neurones of SNpc, which survived until adulthood, become resistant to MPTP toxicity. Stimulation of expression, post-translational modification and aggregation of α-synuclein in neurones of SNpc by MPTP are well documented, but it is less clear how these correlate with neuronal death (Kowall *et al.* 2000; Vila *et al.* 2000; Przedborski *et al.* 2001b; Meredith *et al.* 2002; Kuhn *et al.* 2003). Studies of dopaminergic neurones overexpressing different forms of human α-synuclein produced controversial results – either increased (Richfield *et al.* 2002) or unchanged (Rathke-Hartlieb *et al.* 2001; Dong *et al.* 2002) sensitivity to MPTP toxicity has been demonstrated. Results are more consistent for mice with targeted inactivation of *α-synuclein* gene. Resistance to MPTP toxicity has been shown previously for two independently generated mouse strains with targeted inactivation of *α-synuclein* gene (Dauer *et al.* 2002; Schluter *et al.* 2003) and we have demonstrated the same effect for the third strain. However, we have shown that dopaminergic neurones of SNpc of γ-synuclein and double α-synuclein/γ-synuclein null mutant mice are also resistant to MPTP toxicity. Concurrently, we detected increased levels of β-synuclein in midbrain of adult mice lacking α-, γ- or both synucleins. Neuroprotective effect of β-synuclein overexpression has recently been demonstrated in different experimental systems (Hashimoto *et al.* 2001; da Costa *et al.* 2003) and it is feasible to suggest that compensatory increase of β-synuclein level in midbrain neurones survived beyond the period of NCD makes them less sensitive to certain neurotoxic insults. The levels of β-synuclein are similar in midbrains of α-synuclein and γ-synuclein null mutant mice and loss of both α- and γ-synuclein in double mutant mice is not accompanied by further increase of β-synuclein level. These data are consistent with the discussed above idea that α- and γ-synuclein are involved in the same intracellular processes and a suggestion that increased level of β-synuclein might be a factor required for effective survival of neurones in which these processes are compromised. Further experiments should reveal spatial and developmental patterns of β-synuclein accumulation in brains of mutant mice as well as if and how increased β-synuclein level is linked with activation of pro-survival or inhibition of pro-death mechanisms that might be responsible for resistance of synuclein-deficient SNpc neurones to MPTP.

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